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Investigation into the mechanisms of platelet recruitment and migration during allergic inflammation

Shah, Sajeel Ahmad

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Investigation into the mechanisms of platelet recruitment and migration during allergic inflammation

Sajeel Ahmad Shah

A thesis submitted for the degree of

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Supervisors:

Dr Simon Pitchford and Prof. Clive Page

Sackler Institute of Pulmonary Pharmacology

Institute of Pharmaceutical Science

School of Cancer and Pharmaceutical Sciences

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King's College London

Abstract

A dichotomy in platelet activation has been described, where along with well-known thrombotic and haemostatic responses, platelets can be activated via alternate pathways leading to inflammatory responses. Such activation pathways are believed to be involved in allergic inflammatory diseases such as asthma, where platelets can migrate extravascularly into lung airway walls and parenchyma in response to allergen. Understanding the mechanisms of platelet recruitment and migration in allergic inflammation could lead to novel drug approaches for the treatment of allergic conditions.

The purpose of this research was to firstly develop and advance *in-vitro* and *in-vivo* models of platelet migration in response to allergen sensitization and challenge. Moreover, the mechanisms behind the allergic inflammatory actions of platelets by investigating the roles of platelet chemokine receptors.

Platelet CCR1, CCR3, CCR4 and CXCR4 chemokine receptor expression was confirmed by flow cytometry and western blot analysis, using antibodies specific to each receptor. Several *in-vitro* chemotaxis assays were studied and developed for measuring human and mouse platelet motility. Platelet chemotaxis was recorded *in-vitro* towards eotaxin, fMLP, MDC and SDF-1 α . Furthermore, increased platelet chemotaxis to eotaxin was recorded when platelets were harvested from mice sensitized to the experimental allergen ovalbumin, compared to platelets harvested from sham-sensitized mice.

In other experiments, a house dust mite extract (HDM) sensitisation and exposure protocol was optimised to sensitise mice to allergen, to then be used for: 1. intravital-microscopy preparations of mouse cremaster muscle; 2. lung immunohistochemistry assays. Intravital microscopy of fluorescently labelled platelets in the mouse cremaster muscle enabled recordings of platelet-endothelial wall interactions. After HDM challenge in the

cremaster muscle, increased platelet rolling and adhesion events were recorded in post capillary venules of HDM-sensitised mice compared with sham-sensitised mice. Immunohistochemical staining of extravascular platelets in cremaster muscle sections, and lungs similarly displayed an increased number of platelets in mice that were HDM-sensitised and HDM challenged.

The roles of platelet chemokine CCR1, CCR3, CCR4, and CXCR4 receptors on platelet accumulation were investigated using antagonists to these receptors *in-vivo*. In response to allergen challenge, CCR3 receptor antagonism attenuated localized platelet adhesion and accumulation; whilst CXCR4 receptor antagonism decreased leukocyte migration.

These results demonstrate that platelet migration can occur in response to a chemokine stimulus *in-vitro* and allergen exposure *in-vivo*, and suggests novel roles of platelet chemokine receptors in allergic inflammatory platelet function.

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Publications and conference proceedings

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Table of Contents

| | |
|---|------|
| Abstract..... | II |
| Acknowledgements | IV |
| Publications and conference proceedings..... | V |
| List of Figures | XIII |
| List of Tables..... | XVI |
| List of abbreviations..... | XVII |
| Chapter 1 Introduction..... | 1 |
| 1.1 Platelet function in haemostasis and host defence | 2 |
| 1.1.1 Platelet structure and production | 2 |
| 1.1.2 Platelet aggregation | 4 |
| 1.2 Platelets in allergic inflammation..... | 8 |
| 1.2.1 Asthma..... | 8 |
| 1.2.2 Allergen sensitization and exposure | 9 |
| 1.2.3 Platelet activation in allergic inflammation..... | 11 |
| 1.2.4 Platelets in asthma | 12 |
| 1.3 Platelet recruitment and migration | 20 |
| 1.3.1 Cellular motility and chemotaxis..... | 20 |
| 1.3.2 Can platelets migrate? | 20 |
| 1.3.3 Platelet chemotaxis..... | 21 |
| 1.4 Chemokine receptors..... | 25 |
| 1.4.1 Chemokines and their receptors | 25 |

| | | |
|-----------|---|----|
| 1.4.2 | Chemokine receptors expressed on platelets | 27 |
| 1.4.3 | Chemokine receptor function on platelets and relevance to allergic inflammation. | 28 |
| 1.5 | Aims and objectives | 32 |
| Chapter 2 | Materials/Methods | 33 |
| 2.1 | Materials..... | 34 |
| 2.2 | Buffer preparations..... | 38 |
| 2.3 | <i>In vivo</i> studies..... | 39 |
| 2.3.1 | Animals | 39 |
| 2.3.2 | Mouse sensitization and challenge to OVA | 39 |
| 2.3.3 | Mouse sensitization to HDM..... | 40 |
| 2.3.4 | HDM challenge and bronchoalveolar lavage fluid collection | 40 |
| 2.3.5 | Total cell counts and differential cell quantification | 41 |
| 2.3.6 | Lung harvesting for histology and immunohistochemistry | 41 |
| 2.3.7 | HDM-sensitized mice HDM challenge following an extended recovery period..... | 41 |
| 2.3.8 | Intravital microscopy of mouse cremaster muscle | 42 |
| 2.3.9 | Establishing an allergic inflammatory response in the cremaster muscle..... | 43 |
| 2.3.10 | Cremaster muscle harvesting for histology and immunohistochemistry | 44 |
| 2.3.11 | Chemokine receptor antagonist treatment | 44 |

| | | |
|--------|---|----|
| 2.3.12 | Exogenous eotaxin administration to the scrotum for intravital microscopy | 45 |
| 2.3.13 | Bleeding time measurements following injury | 45 |
| 2.3.14 | Evans blue allergic skin test | 45 |
| 2.4 | <i>In vitro</i> chemotaxis assays | 46 |
| 2.4.1 | Mouse washed platelet preparation | 46 |
| 2.4.2 | Microplate chemotaxis assay for the evaluation of mouse platelet chemotaxis towards different chemokines | 46 |
| 2.4.3 | Characterisation experiments of microfluidic chemotaxis assay to investigate platelet chemotaxis | 47 |
| 2.4.4 | Human washed platelet preparation for in-vitro chemotaxis assay .. | 50 |
| 2.4.5 | Characterisation experiments of transwell chemotaxis assay to measure human platelet motility to chemokines | 50 |
| 2.5 | Chemokine receptor expression | 52 |
| 2.5.1 | Chemokine receptor expression by flow cytometry | 52 |
| 2.5.2 | Total protein assay | 52 |
| 2.5.3 | Measurements of chemokine receptor expression by western blot analysis | 52 |
| 2.6 | Immunohistochemistry and histology | 54 |
| 2.6.1 | Mouse lung and cremaster muscle processing and sectioning | 54 |
| 2.6.2 | Mouse lung and cremaster muscle CD42b platelet stain, and polymorphonuclear cell staining | 54 |
| 2.6.3 | Mouse lung and cremaster muscle Luna stain for eosinophils | 54 |

| | | |
|-----------|--|----|
| 2.6.4 | Quantification of platelets, eosinophils and PMNs in mouse lung and cremaster muscle sections..... | 55 |
| 2.6.5 | Human lung CD42b platelet stain | 55 |
| 2.7 | Statistical analysis | 56 |
| Chapter 3 | Results I: Presence of platelets in human lung samples and expression of platelet chemokine receptors in sham vs allergen-sensitized mice | 57 |
| 3.1 | Immunohistochemical staining of platelets in human lung sections | 58 |
| 3.2 | Platelet chemokine receptor expression in sham and allergen-sensitized mice | 61 |
| 3.2.1 | Platelet chemokine receptor expression in OVA-sensitized mice by flow cytometry | 61 |
| 3.2.2 | Western blot analysis of CCR1, CCR3, CCR4 and CXCR4 platelet chemokine receptors in HDM-sensitized mice..... | 65 |
| Chapter 4 | Results II: The effects of allergen-sensitization on chemokine receptor induced platelet chemotaxis and <i>in-vitro</i> platelet chemotaxis assay model development..... | 68 |
| 4.1 | Microplate chemotaxis assay | 69 |
| 4.1.1 | An investigation into the effects of allergen-sensitization on platelet chemotaxis..... | 69 |
| 4.1.2 | An investigation into the effects of allergen challenge on platelet chemotaxis..... | 71 |
| 4.2 | Transwell chemotaxis assay | 73 |
| 4.2.1 | Platelet chemotaxis towards fMLP using a transwell assay design. | 73 |

| | | |
|-----------|--|-----|
| 4.2.2 | The effects of incubation times on platelet migration in the transwell chemotaxis assay | 75 |
| 4.2.3 | fMLP concentration response with optimized incubation time | 76 |
| 4.2.4 | An investigation into the effects of eotaxin, MDC and SDF-1 α on human platelet chemotaxis | 78 |
| 4.3 | Microfluidic chemotaxis assay characterisation studies | 80 |
| 4.3.1 | Ibidi microslide preparation | 80 |
| 4.3.2 | Microfluidic chemotaxis assay pilot study | 82 |
| 4.3.3 | Effects of time for platelets to settle on the microfluidic chemotaxis assay slide..... | 83 |
| 4.3.4 | Concentration gradient viability study | 86 |
| 4.3.5 | Effect of increasing concentrations of MDC and SDF-1 α | 87 |
| 4.3.6 | Platelet firm adhesion to microfluidic chemotaxis assay slide..... | 91 |
| 4.3.7 | Testing different well coatings for platelet chemotaxis..... | 92 |
| 4.3.8 | Elucidation of the basal movement of platelets in control groups... | 93 |
| Chapter 5 | Results III: Development of a HDM-sensitization and exposure model, to assess platelet recruitment, adhesion and migration in response to allergen challenge | 96 |
| 5.1 | HDM allergen-sensitization model validation | 97 |
| 5.1.1 | Initial studies investigating the effects of OVA-sensitization on intravital cremaster muscle preparations | 97 |
| 5.1.2 | HDM-sensitization protocol development | 98 |
| 5.1.3 | Histological staining of eosinophils in lungs of HDM-sensitized mice | 100 |

| | | |
|-----------|--|-----|
| 5.1.4 | Platelets in extravascular lung compartments of HDM-sensitized mice | 101 |
| 5.1.5 | HDM systemic sensitization investigation | 102 |
| 5.2 | Intravital microscopy protocol development for assessing platelet recruitment in response to allergen challenge | 104 |
| 5.2.1 | Cremaster muscle imaging set up..... | 104 |
| 5.2.2 | Allergic inflammatory response in the cremaster muscle | 105 |
| 5.2.3 | Eosinophil staining of cremaster muscle..... | 106 |
| 5.2.4 | Platelet activity in response to allergen challenge in the cremaster muscle..... | 108 |
| 5.2.5 | Platelet migration to cremaster muscle following allergen challenge | 109 |
| Chapter 6 | Results IV: The roles of chemokine receptors on platelet tissue recruitment and accumulation in response to allergen challenge..... | 112 |
| 6.1 | The effects of platelet chemokine receptor antagonists in the lung | 113 |
| 6.1.1 | Effect of chemokine receptor antagonists on platelet migration following allergen challenge | 113 |
| 6.1.2 | The effects of chemokine receptor antagonists following a one week recovery period..... | 115 |
| 6.1.3 | Bleeding times after chemokine receptor antagonist treatment..... | 118 |
| 6.2 | The effects of platelet chemokine receptor antagonists on platelet recruitment and accumulation to the cremaster muscle following allergen challenge | 120 |

| | | |
|-----------|--|-----|
| 6.2.1 | The effects of platelet chemokine receptor antagonists on leukocyte recruitment to the cremaster muscle..... | 120 |
| 6.2.2 | The effects of chemokine receptor antagonists on intravascular platelet recruitment and accumulation in the cremaster muscle | 121 |
| 6.2.3 | The effects of chemokine receptor antagonists on extravascular platelet accumulation following allergen challenge in the cremaster muscle..... | 124 |
| 6.2.4 | The effects of s.c. eotaxin administration to the scrotum of HDM-sensitized mice | 125 |
| Chapter 7 | Discussion..... | 128 |
| 7.1 | Platelet recruitment in fatal asthmatic patients | 129 |
| 7.2 | <i>In-vitro</i> and <i>in-vivo</i> model development | 132 |
| 7.2.1 | In-vitro platelet chemotaxis assays..... | 132 |
| 7.2.2 | Intravital microscopy of mouse cremaster muscle | 135 |
| 7.3 | Platelet chemokine receptors..... | 139 |
| 7.3.1 | Platelet CCR1 receptor | 139 |
| 7.3.2 | Platelet CCR4 receptor | 140 |
| 7.3.3 | Platelet CXCR4 receptor | 142 |
| 7.3.4 | Platelet CCR3 receptor | 143 |
| 7.4 | Summary | 147 |
| 7.5 | Future work..... | 148 |
| Chapter 8 | References..... | 150 |

List of Figures

| | |
|---|----|
| Figure 1.1: Illustration of the effects of platelet activation in allergic asthma..... | 16 |
| Figure 2.1: Mouse OVA-sensitization and challenge protocol..... | 39 |
| Figure 2.2: Mouse HDM-sensitization protocol..... | 40 |
| Figure 2.3: Mouse HDM-sensitization and i.n. HDM challenge protocol..... | 40 |
| Figure 2.4: Mouse HDM-sensitization and i.n. HDM challenge with extended protocol. | 41 |
| Figure 2.5: Illustration of mouse cremaster muscle preparation for intravital microscopy. .. | 42 |
| Figure 2.6: Mouse HDM-sensitization and HDM s.c. challenge protocol..... | 44 |
| Figure 2.7: Microplate chemotaxis assay arrangement..... | 47 |
| Figure 2.8: Diagrammatic representation of microfluidic chemotaxis assay..... | 48 |
| Figure 2.9: Transwell chemotaxis assay arrangement. | 50 |
| Figure 2.8: HTC transwell assay arrangement..... | 51 |
| Figure 3.1: Immunostaining CD42b+ platelets in human lung samples..... | 59 |
| Figure 3.2: Representative immunostaining of CD42b+ platelets in airway walls..... | 60 |
| Figure 3.3: Representative flow cytometry traces of isotype IgG-PE and CCR1-PE..... | 62 |
| Figure 3.4: Representative flow cytometry traces of CCR3-PE, CCR4-PE and CXCR4-PE. | 63 |
| Figure 3.5: The effects of OVA-sensitization of mice on platelet chemokine receptor expression and MFI..... | 64 |
| Figure 3.6: Representative immunoblots of CCR1, CCR3, CCR4 and CXCR4 receptor expression..... | 65 |
| Figure 3.7: The effects of HDM-sensitization of mice on platelet chemokine receptor expression..... | 66 |
| Figure 4.1: The effects of MIP-1 α , eotaxin, MDC, and SDF-1 α on platelet chemotaxis in OVA-sensitized mice. | 70 |

| | |
|---|-----|
| Figure 4.2: The effects of MIP-1 α , eotaxin, MDC, and SDF-1 α on platelet chemotaxis in OVA-sensitized and OVA-challenged mice..... | 72 |
| Figure 4.3: Human platelet chemotaxis fMLP concentration response. | 74 |
| Figure 4.4: The effects of different incubation times on platelet chemotaxis to fMLP. | 76 |
| Figure 4.5: fMLP concentration response in optimized transwell chemotaxis assay. | 77 |
| Figure 4.6: The effects of eotaxin, MDC and SDF-1 α on human platelet chemotaxis..... | 79 |
| Figure 4.7: Example of platelet tracking in the microfluidic chemotaxis assay. | 81 |
| Figure 4.8: Microfluidic chemotaxis assay pilot study spider plots..... | 82 |
| Figure 4.8: The effects of increased platelet settling time on platelet chemotaxis. | 85 |
| Figure 4.9: The effect of time on the concentration gradient viability in the microfluidic chemotaxis assay. | 87 |
| Figure 4.10: Concentration response to MDC in the microfluidic chemotaxis assay..... | 89 |
| Figure 4.11: Concentrations response to SDF-1 α in the microfluidic chemotaxis assay..... | 90 |
| Figure 4.13: Platelet adhesion to the surface of the microfluidic chemotaxis assay slide. | 91 |
| Figure 4.14: The effects of different well coatings on platelet chemotaxis. | 93 |
| Figure 4.15: Elucidation of control drifting effect. | 94 |
| Figure 5.1: Aluminium hydroxide deposits in the scrotum..... | 98 |
| Figure 5.2: The effects of HDM-sensitization on pulmonary leukocyte recruitment. | 99 |
| Figure 5.3: The effects of HDM-sensitization on eosinophil recruitment to the airway walls. | 100 |
| Figure 5.4: The effects of HDM-sensitization on platelet recruitment to the airway walls. | 101 |
| Figure 5.5: The effects allergen exposure on s.c. oedema formation..... | 103 |
| Figure 5.6: Representative images of cremaster muscle in bright field light, 580 nm fluorescence light and merged displays..... | 104 |
| Figure 5.7: The effects of s.c. HDM challenge on leukocyte migration to the cremaster muscle..... | 105 |
| Figure 5.8: The effects of s.c. HDM challenge in HDM-sensitized mice on..... | 107 |

| | |
|---|-----|
| eosinophil migration to the cremaster muscle. | 107 |
| Figure 5.9: The effects of s.c. HDM challenge in HDM-sensitized mice on intravascular platelet adhesion and platelet rolling events. | 109 |
| Figure 5.10: The effects of HDM-sensitization and HDM challenge on platelet migration to the cremaster muscle. | 111 |
| Figure 6.1: SB328437, C-021 and AMD3100 chemical structures. | 114 |
| Figure 6.2: The effects of chemokine receptor antagonists on platelet migration following allergen challenge. | 115 |
| Figure 6.3: The effects of chemokine receptor antagonists on platelet migration after allergen challenge, following a recovery period. | 116 |
| Figure 6.4: The effects of chemokine receptor antagonists on PMN migration after allergen challenge. | 118 |
| Figure 6.5: The effects of chemokine receptor antagonists on bleeding times following allergen challenge after a recovery period. | 119 |
| Figure 6.6: The effects of chemokine receptor antagonists on leukocyte accumulation following allergen challenge in the cremaster muscle. | 121 |
| Figure 6.7: The effects of chemokine receptor antagonists on platelet adhesion and platelet rolling events in the cremaster muscle. | 123 |
| Figure 6.8: The effects of chemokine receptor antagonists on platelet migration following allergen challenge in the cremaster muscle. | 124 |
| Figure 6.9: The effects of eotaxin on leukocyte recruitment, platelet adhesion and platelet rolling events in the cremaster muscle. | 126 |

List of Tables

| | |
|---|----|
| Table 1: Platelet granule constituents adapted from Smyth et al., 2009 and Amison 2014..... | 3 |
| Table 2: Human chemokines and their receptors..... | 25 |
| Table 3: Measurements of platelet motility from pilot microfluidic chemotaxis assay study. | 83 |
| Table 4: Measurements of platelet motility from platelet settling time study. | 84 |
| Table 5: Measurements of platelet motility from concentration gradient viability study. | 86 |
| Table 6: Measurements of platelet motility in concentration response to MDC study..... | 88 |
| Table 7: Measurements of platelet motility in concentration response to SDF-1 α study. | 88 |

List of abbreviations

| | |
|---------|--|
| 12-HETE | 12-hydroxyeicosatetraenoic acid |
| 5-HT | 5-hydroxytryptamine |
| ABCD-1 | ATP-binding cassette, sub-family D, member 1, CCL22 |
| ABCD-2 | ATP-binding cassette, sub-family D, member 2, CCL17 |
| ABCD-3 | ATP-binding cassette, sub-family D, member 3, CX3CL1 |
| ACD | Acid citrate dextrose |
| ADP | Adenosine 5'-diphosphate |
| AERD | Aspirin-induced exacerbated respiratory disease |
| AHR | Airway hyperresponsiveness |
| AMAC-1 | Alternative macrophage activation-associated CC chemokine-1, CCL18 |
| ANOVA | Analysis of variance |
| APC | Antigen presenting cells |
| ASMC | Airway smooth muscle cell |
| ATAC | Activation-induced T cell-derived and chemokine-related molecule, XCL1 |
| ATP | Adenosine 5'-triphosphate |
| BAL | Bronchoalveolar lavage |
| BCA | Bicinchoninic acid |
| BCA1 | B cell-attracting chemokine 1, CXCL13 |
| Beta-TG | Beta-thomboglobulin, CXCL7 |
| BLC | B lymphocyte chemoattractant, CXCL13 |
| BRAK | Breast and kidney-expressed chemokine, CXCL14 |

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| BSA | Bovine serum albumin |
| CRG-2 | Cytokine responsive gene 2, CXCL10 |
| CTACK | Cutaneous T-cell-attracting chemokine, CCL27 |
| CTAP-III | Connective tissue-activating peptide III, CXCL7 |
| CKC | Chemokinetic control group |
| DAB | 3,3'-diaminobenzidine |
| DAG | Diacyl-glycerol |
| DC-CK1 | Dendritic cell-specific CC-chemokine 1, CCL18 |
| DMC | Dendritic and monocyte chemokine-like protein, CXCL17 |
| DMSO | Dimethyl sulfoxide |
| DPX | Depex |
| ELC | EBI1 ligand chemokine, CCL19 |
| ENA-78 | Epithelial cell-derived neutrophil-activating-78, CXCL5 |
| EPO | Eosinophil peroxidase |
| F | Factor |
| FITC | Fluorescein isothiocyanate |
| fMLP | N-formyl-methionyl-leucyl phenylalanine |
| FPR | N-formyl peptide receptors |
| GCP-2 | Granulocyte chemotactic protein-2, CXCL6 |
| GDP | Guanosine 5'-diphosphate |
| GMP 33 | Granule membrane protein 33 |
| GP | Glycoprotein |
| GPCR | G-protein coupled receptors |

| | |
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| Gro- α | Gro, growth-related oncogene α , CXCL1 |
| Gro- β | Gro, growth-related oncogene β , CXCL2 |
| Gro- γ | Gro, growth-related oncogene γ , CXCL3 |
| HCC-1 | Hemofiltrate CC Chemokine 1, CCL14 |
| HCC-2 | Hemofiltrate CC Chemokine 2, CCL15 |
| HCC-4 | Hemofiltrate CC Chemokine 4, CCL16 |
| HDM | <i>Pteronyssinus</i> extract |
| HEPES | (4-(2-hydroxyethyl)-1piperazineethanesulfonic acid) |
| HRP | Horseradish peroxidase |
| I-TAC | Interferon-inducible T-cell alpha chemoattractant, CXCL11 |
| IgE | Immunoglobulin E |
| IgG | Immunoglobulin G |
| IL | Interleukin |
| IP10 | Interferon gamma-induced protein 10, CXCL10 |
| ILC | IL-11 R-alpha-locus chemokine, CCL27 |
| KC | Keratinocyte chemoattractant, CXCL1 |
| LAG-1 | Lymphocyte activation gene, CCL4L1 and CCL4L2 |
| LARC | Liver activation-regulated chemokine, CCL20 |
| LEC | Liver-expressed chemokine, CCL16 |
| LDL | Lithium dodecyl sulfate |
| LPS | Lipopolysaccharide |
| MAD | Mean accumulated distance |
| MBP | Major basic protein |

| | |
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| MCAF | Monocyte chemotactic and activating factor, CCL2 |
| MCP-1 | Monocyte chemoattractant protein 1, CCL2 |
| MCP-2 | Monocyte chemoattractant protein 2, CCL8 |
| MCP-3 | Monocyte chemoattractant protein 3, CCL7 |
| MCP-4 | Monocyte chemoattractant protein 4, CCL13 |
| MDC | Macrophage derived chemokine, CCL22 |
| MEC | Mucosae-associated epithelial chemokine, CCL28 |
| MED | Mean Euclidean distance |
| MGSA- α | Melanoma growth stimulating activity- α , CXCL1 |
| MGSA- β | Melanoma growth stimulating activity- β , CXCL2 |
| MGSA- γ | Melanoma growth stimulating activity- γ , CXCL3 |
| MFI | Mean fluorescence intensity |
| MIF | Migration inhibitory factor |
| MIG | Monokine induced by gamma interferon, CXCL9 |
| MIP-1 α | Macrophage inflammatory protein 1 α , CCL3 |
| MIP-1 β | Macrophage inflammatory protein 1 β , CCL4 |
| MIP-2 | Macrophage inflammatory protein 2, CXCL1 |
| MIP-2 α | Macrophage inflammatory protein 2 α , CXCL2 |
| MIP-2 β | Macrophage inflammatory protein 2 β , CXCL3 |
| MIP-3 α | Macrophage inflammatory protein 3 α , CCL20 |
| MIP-3 β | Macrophage inflammatory protein 3 β , CCL19 |
| MIP-4 α | Macrophage inflammatory protein 4 α , CCL26 |
| MOPS | 3-(N-morpholino)propanesulfonic acid |

| | |
|------------------|--|
| MPIF-1 | Myeloid progenitor inhibitory factor 1, CCL23 |
| MPIF-2 | Myeloid progenitor inhibitory factor 2, CCL24 |
| Munc13-4 | Mammalian homolog of <i>C. elegans</i> uncoordinated gene 13-4 |
| NAP-2 | Neutrophil activation protein-2, CXCL7 |
| NFP | N-formylated peptides |
| OVA | Ovalbumin |
| PAF | Platelet-activating factor |
| PARC | Pulmonary and activation-regulated chemokine, CCL18 |
| PBS | Phosphate buffered saline |
| PE | Phycoerythrin |
| PF4 | Platelet factor 4, CXCL4 |
| PFA | Paraformaldehyde |
| PGE ₁ | Prostaglandin E1 |
| PGI ₂ | Prostacyclin (prostaglandin I ₂) |
| PKA | Protein kinase A |
| PLC | Phospholipase C |
| PMN | Polymorphonuclear neutrophils |
| PPP | Platelet poor plasma |
| PRP | Platelet rich plasma |
| PSGL-1 | P-selectin glycoprotein ligand 1 |
| RANTES | Regulated upon activation normally T-cell expressed and secreted, CCL5 |
| RBL | Rat basophilic leukaemia |
| RPMI | Roswell Park Memorial Institute |

| | |
|----------------|--|
| SCM-1 α | Single C motif-1 α , XCL1 |
| SCM-1 β | Single C motif-1 β , XCL2 |
| SD | Standard deviation |
| SDF-1 α | Stromal cell derived factor-1 α , CXCL12 |
| SDF-1 β | Stromal cell derived factor-1 β , CXCL12 |
| SDF-1 γ | Stromal cell derived factor-1 γ , CXCL12 |
| SEM | Standard error of the mean |
| SLC | Secondary lymphoid-tissue chemokine, CCL21 |
| SR-PSOX | Scavenger receptor phosphatidylserine and oxidized lipoprotein, CXCL16 |
| STCP-1 | Stimulated T cell chemoattractant protein-1, CCL22 |
| TARC | Thymus activation regulated chemokine, CCL17 |
| TBST | Tris-buffered saline with Tween 20 |
| TCA3 | T cell activation gene 3, CCL1 |
| TECK | Thymus-Expressed Chemokine, CCL25 |
| TF | Tissue factor |
| TLR | Toll-like receptor |
| WT | Wild type |

Chapter 1 Introduction

1.1 Platelet function in haemostasis and host defence

1.1.1 Platelet structure and production

Small, anuclear, spherical cell fragments called platelets, were first described in the 19th century, along with their vital roles in haemostasis and thrombosis (Osler, 1874; Bizzozero, 1881). Under normal resting conditions, human platelets are roughly 3 μm at the widest point with a blood concentration of $1.5\text{--}3 \times 10^8$ platelets/mL (Hartwig and Italiano, 2003). Platelets are produced by the fragmentation of large polyploid megakaryocytes, in a process termed thrombopoiesis, and released into the circulation where they survive for 7–10 days (Hartwig and Italiano, 2003). Platelet production was believed to be restricted to the bone marrow. However, more recent evidence suggests that megakaryocytes can mature in the lung and release platelets into the pulmonary circulation, contributing an estimated 50% to the total platelet production (Wang et al., 2015; Lefrançois et al., 2017).

Platelets do not replicate or include nuclei, but contain mRNA which they are able to translate and independently produce proteins, to supplement proteins produced by megakaryocytes (Yeaman, 2014). Platelets contain α -granules, dense δ -granules and lysosomal λ -granules, which can release a broad range of over 300 different proteins following degranulation (Coppinger et al., 2004), examples of which are displayed in Table 1. The secretion of such mediators from platelets are fundamentally important in platelet function (Smyth et al., 2009). Aging platelets are eventually cleared from the circulation through an intrinsic apoptosis mechanism in the liver and spleen (Kile, 2014).

Table 1: Platelet granule constituents adapted from Smyth et al., 2009 and Amison 2014

| α -granules | Dense δ -granules | Lysosomal λ -granules |
|---|---|--|
| Adhesive glycoproteins <ul style="list-style-type: none"> • Fibrinogen • von Willebrand Factor • von Willebrand Factor propetide • Fibronectin • Thrombospondin-1 • Vitronectin Coagulation Factors <ul style="list-style-type: none"> • Factor V • Protein S • Factor XI Mitogenic Factors <ul style="list-style-type: none"> • Platelet-derived growth factor • Transforming growth factor-β • Endothelial cell growth factor • Epidermal growth factor • Insulin-like growth factor-1 Angiogenic factors <ul style="list-style-type: none"> • Vascular endothelial growth factor • Platelet factor 4 Fibrinolytic inhibitors <ul style="list-style-type: none"> • α2-Plasmin inhibitor • Plasminogen activator inhibitor-1 Multimerin | ATP ADP Calcium Serotonin Pyrophosphate GDP Magnesium Other secreted/released proteins <ul style="list-style-type: none"> • Protease nexin 1 • Gas6 • Amyloid β-protein precursor • Tissue factor pathway inhibitor • Factor XIII • α1-Protease inhibitor • Complement 1 inhibitor • High molecular weight kininogen • α2-Macroglobulin • Vascular permeability factor • Interleukin-1b • Histidine-rich glycoprotein • Sphingosine-1-phosphate Chemokines <ul style="list-style-type: none"> • MIP-1α (CCL3) • RANTES (CCL5) • MCP-3 (CCL7) • Gro-α (CXCL1) • ENA-78 (CXCL5) • NAP-2 (CXCL7) • Interleukin-8 (CXCL8) • TARC (CCL17) | CD63 (LAMP-3) Cathepsin D Cathepsin E Proline Carboxypeptidase A Proline Carboxypeptidase B β -N-acetylhexosaminidase β -D-glucuronidase β -D-galactosidase α -D-mannosidase α -L-arabinofuranosidase Acid phosphatase Arylsulphatase |
| Platelet factor 4 (CXCL4) Immunoglobulins β -defensin 2 Thymosin β 4 Granule membrane-specific proteins <ul style="list-style-type: none"> • P-selectin (CD62P) • GMP 33 • GPIb/IX/V • GPIIb/IIIa • GPIV | | |

1.1.2 Platelet aggregation

The well-established mechanisms of platelet aggregation are critical for maintaining blood circulation by preventing haemorrhage. Circulating resting platelets are modulated by endothelial cells which promote protein C to prevent thrombin formation, and release prostaglandin I₂ and nitric oxide that inhibit platelet function (Versteeg et al., 2013). Reciprocally, platelets help to maintain endothelial ultrastructure, promote endothelial cell growth and enhance barrier function of endothelial cells, by release of mediators such as endothelial cell growth factor, adenosine 5'-diphosphate (ADP), serotonin and sphingosine-1-phosphate (Saba and Mason, 1975; King and Buchwald, 1984; Schaphorst et al., 2003; Ho-Tin-Noé et al., 2011).

Following vessel rupture, endothelial cells become damaged and platelets become exposed to the subendothelial matrix. Platelets bind von Willebrand factor through glycoprotein (GP) Ib α and bind collagen through GPVI and integrin $\alpha_2\beta_1$, expressed on the subendothelial matrix. These adhesive events cause adhesion of platelets to the exposed damaged area, which along with thrombin, can initiate platelet activation. Consequently, the clotting cascade is initiated, with the first phase (initiation, or extrinsic phase) involving blood borne clotting factors acting on cells in the extravascular tissue that express tissue factor (TF). TF binds to factor (F) VII and triggers a cascade of clotting factors resulting in FXa converting FII (prothrombin) to FIIa (thrombin), thrombin then converts fibrinogen to fibrin. The amplification (intrinsic) phase is initiated by thrombin on activated platelets and involves a series of clotting factors that leads to further thrombin formation (Brass, 2010; Yeaman, 2014).

Activation of platelets in aggregatory responses leads to shape change, granule release and surface expression of adhesion molecules and receptors (Gear and Polanowska-Grabowska, 2002). Central to these aggregatory effects of platelets is the release of ADP, from dense δ -granules, which binds to P2Y₁ and P2Y₁₂ receptors; conformational changes of GPIIb/IIIa fibrinogen receptors and increased surface expression of GPIIb/IIIa receptors and mediation of GPIIb/IIIa expression by thromboxane A₂ (TXA₂), which also causes

vasoconstriction. These changes in platelets lead to the formation of a cross-linked, elastic fibrin clot over the area of injury, which can be attenuated by proteolytic feedback. This protective mechanism is a vitally important physiological process that prevents bleeding and enables wound repair (Smyth et al., 2009; Brass, 2010; Versteeg et al., 2013).

1.1.3. Platelets and host defence

The role of platelets in thrombosis and haemostasis is well understood and has led to the discovery of effective anti-platelet drugs to treat thrombosis. However, platelets are also important for host defence and inflammatory responses, which are distinct physiological processes to that of thrombosis and haemostasis (Page and Pitchford, 2014). The difference in platelet function in haemostasis compared with inflammation has led to the hypothesis that there is a dichotomy in platelet activation (Page, 1988), which has recently been confirmed experimentally using murine models of allergic airways disease (discussed below) (Amison et al., 2015; Pan et al., 2015). Indeed, platelets have been shown to be important in host defence against microbial infections (van den Boogaard et al., 2015; Hurley et al., 2016).

Platelets are the first responders to sites of tissue injury, endothelial injury and infection, leading to haemostatic responses of platelets and key roles in the early intervention against infection. Platelets can target sites of bacteria-specific proteins through activation via complement C3a and C5a receptors (del Conde et al., 2005; Yeaman, 2010), and have been shown to undergo chemotaxis to exposed collagen through interactions with plasma factors (Lowenhaupt, 1978; Lowenhaupt et al., 1982). Furthermore, platelets can become activated in response to bacterial N-formylated peptides (NFP) via N-formyl peptide receptors (FPR) that are expressed on their surface (Czapiga et al., 2005). Activation via NFP leads to calcium mobilization, cytoskeletal rearrangements and chemotaxis (Czapiga et al., 2005). Therefore platelets have the potential to accumulate at sites of injury or infection, and initiate antimicrobial actions.

Platelets can detect microbial threats through the expression of receptors that recognise pathogen-associated molecular patterns. Platelets upregulate toll-like receptor (TLR)1 and TLR6 in vascular lesions that may involve bacterial infection (Shiraki et al., 2004). Platelets also constitutively express TLR2, TLR4 and TLR9, which have been suggested to have important roles in pathogen recognition (Aslam et al., 2006). Indeed TLR4 dependent signalling pathways are activated in response to lipopolysaccharide (LPS), which have been suggested to interact with the LPS receptor-signalling complex on platelets (Berthet et al., 2012). Furthermore, platelet activation induced by high thrombin concentrations leads to increased TLR9 expression (Aslam et al., 2006). The expression of TLR receptors on platelets enable the detection of bacterial infections, which trigger signalling cascades important in host defence.

Self-amplification of platelet responses occur through autocrine mechanisms involving ADP release from δ -granules, which activates P2Y₁ and P2Y₁₂ receptors leading to further degranulation and release of antimicrobial effector molecules. Platelets have been shown to internalise infectious microorganisms via phagocytosis, aiding the clearance of microorganisms (Youssefian et al., 2002). Platelets contain several direct acting antimicrobial proteins such as human β -defensin 2, CXCL4 (platelet factor 4, PF4), CXCL7 (β -thromboglobulin, beta-TG), hydrogen peroxide and thymosin β 4, which can be released upon platelet activation through degranulation (Tang et al., 2002). Platelet activation in response to microbial infection causes the release of chemokines such as PF4 and CCL5 (regulated upon activation normally T-cell expressed and secreted, RANTES) (Antczak et al., 2011; McMorran et al., 2012). In response to microbial infection, platelet activation can enhance neutrophil responses through direct interactions via platelet CD62P (P-selectin) binding to neutrophil CD162 (P-selectin glycoprotein ligand 1, PSGL-1) (Pan et al., 2015; Zuchtriegel et al., 2016). Once recruited, activated neutrophils can phagocytose pathogens and produce neutrophil extracellular traps, leading to the killing and digestion of pathogens. Platelets

therefore have important roles in the innate immune responses to foreign microorganisms, away from typical platelet aggregatory responses.

1.2 Platelets in allergic inflammation

1.2.1 *Asthma*

Asthma is a chronic allergic inflammatory disease of the airways, which affects 5.4 million people in the UK. It is estimated that every 10 seconds someone has a potentially life threatening asthma attack. In 2016, 1,410 people died from asthma with two thirds of these deaths preventable. Hospital treatment of patients with asthma costs the NHS roughly 1 billion pounds a year (Asthma UK). Thus, there is a clear unmet clinical need for new therapeutic treatments for asthmatic patients.

Patients with asthma suffer from symptoms such as shortness of breath, wheezing, chest tightness, and coughing. In acute asthma these symptoms worsen, and when patients do not respond to standard treatments in cases of severe asthma, this can lead to airway obstruction and respiratory arrest. The typical response to allergen challenge in asthmatic patients in the clinical laboratory involves an acute inflammatory response comprising of early and late phase responses, which are characterized by bronchoconstriction, inflammatory cell recruitment and mucus production. Following the acute phase response, a chronic inflammatory response can be initiated, involving persistent inflammatory cell recruitment to the lung resulting in airway wall remodelling (Barnes, 2011). This model has been widely used to investigate the mechanisms of asthma and to evaluate treatments and many animal models of allergic asthma are design to recapitulate this clinical scenario.

In allergic asthma, patients are predisposed to react to perceived noxious inhaled allergens or other irritants including exercise, cold air and chemical irritants. Classically, within minutes of exposure to allergen the early phase response is initiated. This involves the activation of resident airway immune cells (for example mast cells), by allergen causing cross linking of immunoglobulin E (IgE) which bind to FcεRI receptors, leading to proinflammatory mediator release that cause bronchoconstriction and vasodilation (Barnes, 2011). The late phase response becomes evident 2-9 hours after the initial allergen exposure. Late phase

responses are characterized by leukocyte recruitment (including eosinophils, T cells and neutrophils) to the airways, leading to additional inflammatory mediator release, resulting in further bronchoconstriction, vasodilation and mucus secretion. This is followed by a chronic inflammatory response, which is associated with a T helper 2 cells (Th2) adaptive immune response, causing further tissue injury, and is associated with inappropriate repair mechanisms in the lung, leading to airway remodelling, changes in airway architecture that contributes over time to a loss of respiratory function if not appropriately treated.

There is no cure for allergic asthma, but current treatments include reliever therapies which treat the symptoms as they arise, preventer therapies which reduce the risk of symptoms occurring and combination therapies that help stop symptoms occurring and provide long lasting relief e.g. salmeterol (β_2 agonist) with fluticasone propionate (corticosteroid). However, 5-10% of asthma patients are classified as insensitive or poorly responsive to corticosteroid treatments (Durham et al., 2011). Furthermore, long term corticosteroid treatment is associated with unwanted side effects such as oral candidiasis, pneumonia, cough and contact hypersensitivity. There are also major issues with patient compliance with inhaled therapies, therefore there is a clear unmet clinical need for improved treatments for asthma (Baddar et al., 2014; Ye et al., 2017).

1.2.2 Allergen sensitization and exposure

Sensitization to allergen is a mechanism involving genetic, environmental and immunological factors. This complex interplay between various factors makes association, linkage and predicting the predisposition of a patient to allergic disease challenging. The immunological factors involved in mucosal exposure to allergen are better understood, which begins with the epithelium acting as a physical barrier to inhaled allergen, pathogens, and pollutants. Epithelial cells control movement across the membrane through tight junctions, transporters and selective permeability to ions, which along with epithelial secretions, help maintain the mucus barrier. Epithelial cells also express innate immune receptors that enable detection of foreign viruses, bacteria and fungi, which can induce immune cells to cause an

inflammatory response (Van Ree et al., 2014). Indeed, allergen exposure can activate epithelial cells leading to CCL2 (monocyte chemoattractant protein 1) and CCL20 (macrophage inflammatory protein-3 α) chemokine release, which can recruit and activate dendritic cells (Hammad et al., 2009). Allergens can also bypass the barrier function of epithelial cells through various mechanisms, for example, house dust mite allergen has protease activity, which interferes with tight junctions and facilitates transepithelial allergen delivery (Wan et al., 1999; Van Ree et al., 2014).

Following passage through the epithelial layer, allergens are recognized by dendritic cells or other cells capable of antigen presentation (APCs: antigen presenting cells) which reside in the basolateral layer. APCs subsequently undergo maturation and migration to the secondary lymphatic system. In regards to house dust mite allergen, antigen presenting CD11b⁺ dendritic cells then process and present antigens to T cells via major histocompatibility complex II (Plantinga et al., 2011; Lambrecht and Hammad, 2012). This leads to a Th2 mediated allergic inflammatory response, involving the secretion of cytokines interleukin (IL)-4, IL-5 and IL-13, and B cell immunoglobulin isotype switching. CD4⁺ T cells interact with B cells via CD40 and its ligand (CD40L), and stimulation with IL-4 and IL-13, to cause an isotype switch from immunoglobulin G (IgG) to IgE production (Van Ree et al., 2014). Upon subsequent allergen exposure, allergen is recognised by allergen specific IgE, which is crosslinked with Fc ϵ RI receptors on the surface of lung mast cells and basophils leading to degranulation and release of proinflammatory mediators, for example histamine, 5-hydroxytryptamine (5-HT), prostaglandins, and leukotrienes, contributing to allergic inflammatory responses and bronchospasm during the early phase response, that occurs within seconds and can last for 30 minutes to 1 hour (Stone et al., 2010). Resident immune cells become activated during this period to release various effector cytokines and chemokines to contribute to a late phase response, which is defined by inflammatory cell recruitment to the airways and the release of mucus, leading to further bronchospasm. One example of cytokine release during this period is IL-5 which causes the activation and differentiation of eosinophils

within the bone marrow, leading to their release into the circulation and trafficking to the inflamed tissue (Stone et al., 2010).

1.2.3 Platelet activation in allergic inflammation

Platelets have long been suggested to have an important role in allergic inflammatory diseases (Benveniste et al., 1972). Indeed, platelets possess the requisite components to act as inflammatory cells in the context of allergic inflammation. Intracellular platelet granules can be released upon activation by stimuli emanating from an allergic inflammatory response and they contain several inflammatory mediators such as free radical species, 5-HT, interleukin-1 β (IL-1 β) (Lindemann et al., 2001; Denis et al., 2005), PF4 (Hayashi et al., 1994), RANTES, CXCL7 (Neutrophil activation protein-2, NAP-2; itself a product of processed platelet β -thromboglobulin) and CCL17 (thymus activation regulated chemokine, TARC) (Oliveira and Lukacs, 2003; Smyth et al., 2009). Platelets also express a number of functional receptors which could have roles in allergic inflammatory responses, including immunoglobulin receptors (Fc γ RI, Fc γ RII, Fc γ RIII; Fc ϵ RI, Fc ϵ RII, Fc α RI/CD89) (Joseph et al., 1997; Hasegawa and Matsubara, 2001; Pitchford et al., 2008; Semple and Freedman, 2010), chemokine receptors (CCR1, CCR3, CCR4 and CXCR4) (Clemetson et al., 2000; Kowalska et al., 2000; Abi-Younes et al., 2001), adhesion molecules (P-selectin, PSGL-1 and ICAM2) (Pitchford et al., 2005; Semple and Freedman, 2010) and toll-like receptors (TLR2, TLR4 and TLR9) (Cognasse et al., 2015), which are discussed in this chapter. Thus, these inflammatory mediators and receptors may contribute to allergic inflammatory responses, which have been observed to be released, or activated via IgE-/Fc ϵ RI receptor activation of platelets.

The roles of platelet IgG receptors Fc γ RI, Fc γ RII, Fc γ RIII and Fc α RI/CD89, have not been studied in great depth, although the functionality of the high affinity (Fc ϵ RI) and low affinity (Fc ϵ RII) platelet IgE receptors in response to allergen has been demonstrated. Platelets from patients with asthma, and mice sensitized to experimental allergen were shown to express Fc ϵ RI and Fc ϵ RII (Hasegawa and Matsubara, 2001; Pitchford et al., 2008). Human platelet Fc ϵ RI and Fc ϵ RII receptors are not expressed on all platelets from a given donor, but rather a

distinct population (Pitchford et al., 2008). Platelet activation via FcεRI and FcεRII stimulation leads to the release of cytotoxic mediators, such as reactive oxygen metabolites, free radicals and cationic proteins (Joseph et al., 1986, 1997). The significance of this release compared to cytotoxic mediator release from other allergic inflammatory cells is unknown, however platelet derived cytotoxic mediators have been shown to be critical in the killing of certain types of parasitic infections (Joseph et al., 1986, 1997).

The activation of platelets via IgE can also lead to degranulation of platelet granules, leading to the release of chemokines such as RANTES and PF4, which can recruit leukocytes (Chihara and Nakajima, 1989; Kameyoshi et al., 1992, 1994; Burgers et al., 1993; Chihara et al., 1994). Platelet activation accompanies allergen exposure in patients with asthma, suggesting a IgE-/FcεRI mediated response on platelets occurs (Denis et al., 2005). Moreover, in patients sensitized to the allergen Der p 1, exposure to synthetic Der p 1 was found to activate platelets, but synthetic Der p 1 had no effect on platelets from healthy subjects or on platelets from patients not allergic to Der p 1 (Cardot et al., 1992). Therefore these studies suggest that platelet activation in allergic inflammation is IgE dependant and allergen specific. The ability of platelets to act as inflammatory cells has been demonstrated in allergic inflammatory diseases, such as eczema and allergic rhinitis, and more extensively in asthma which is discussed below.

1.2.4 Platelets in asthma

In patients with asthma, the storage of mediators in circulating platelets is diminished and there is a lack of responsiveness of platelets to aggregatory stimuli *ex-vivo* (Maccia et al., 1977; Palma-Carlos et al., 1991). These platelets have been described to be in an ‘exhausted’ state, where platelets had previously become activated *in-vivo*, leading to release of mediators, and were subsequently refractory to further activation (Maccia et al., 1977; Gallagher et al., 1978; Pareti et al., 1980; Palma-Carlos et al., 1991; Gresele et al., 1993; Page and Pitchford, 2014). Such alterations in platelet activity have been observed in peripheral blood of allergic patients in allergy season, where platelet aggregatory responses were impaired (Gallagher et

al., 1978); similarly, patients with asthma had delayed thrombin generation (Szczeklik et al., 1986; Ind, 1991). Furthermore, platelet lifespans in stable atopic asthmatic patients have been reported to decrease from 8.9 days to 4.7 days (Taytard et al., 1986), suggesting a continual consumption of platelets, presumably because of the ‘exhausted’ platelet state in asthmatic patients.

Following allergen provocation in allergic patients, a mild thrombocytopenia has been reported, which can persist for several hours (Maestrelli et al., 1990; Sullivan et al., 2000; Kowal et al., 2006). Platelet counts taken from asthmatic patients following allergen challenge reduced during the late phase response and lasted up to 24 hours (Sullivan et al., 2000). Also in patients with asthma, 30 minutes following house dust mite or grass pollen challenge, circulating platelet numbers decreased and returned to baseline after 4 hours (Maestrelli et al., 1990). Similarly, Kowal and colleagues demonstrated that 30 minutes following intrabronchial house dust mite extract challenge, circulating platelet counts decreased in asthmatic patients compared to healthy subjects, which gradually returned to baseline (Kowal et al., 2006). Furthermore, Kowal and colleagues investigated levels of platelet plasma markers, 30 minutes after allergen exposure, which showed elevated PF4, beta-TG and soluble P-selectin levels (Kowal et al., 2006). It has therefore been suggested that platelets get recruited to the lungs in asthmatic patients, to account for the mild peripheral thrombocytopenia and the increased levels of platelet markers following allergen challenge (Maestrelli et al., 1990; Sullivan et al., 2000; Kowal et al., 2006; Page and Pitchford, 2014). In accordance with this, platelets are found in the bronchoalveolar lavage fluid of patients with asthma and in extravascular compartments of lungs from bronchial biopsy samples of asthmatic patients (Metzger et al., 1987; Jeffery et al., 1989). Additionally, elevated levels of mediators derived from platelets such as RANTES, free radical species and 5-HT, are measured following bronchial provocation in patients with asthma (Joseph et al., 1983; Hasegawa and Matsubara, 2001; Dürk et al., 2013). Furthermore, immature and mature megakaryocytes are found in extravascular regions of the lung, which may directly contribute

to platelet production (Lefrançois et al., 2017). Indeed, formative studies by Martin and Trowbridge suggested that the process of megakaryocyte fragmentation to produce platelets (Trowbridge et al., 1982; Trowbridge and Martin, 1983), might be affected by inflammatory trauma of the airways, and lead to changes in platelets volume, mass and behaviour (Martin et al., 1983). Increased megakaryocyte trapping in the lungs has been reported in patients who have died of *status asthmaticus*, but it is not known if these events led to the production of platelets with altered behaviour that then influenced disease progression (Aschoff, 1893; Martin et al., 1983).

CD11c⁺ dendritic cells are involved in antigen presentation following exposure to house dust mite and other allergens (Lambrecht and Hammad, 2012). In ovalbumin (OVA)-sensitized mice and after subsequent allergen challenge, increased co-localization of platelets with CD11c⁺ cells was recorded in the airways (Amison et al., 2018a). When platelets were depleted (95% reduction in circulating platelets) during the sensitization period, following allergen challenge, IgE and IL-4 synthesis were suppressed, and pulmonary recruitment of CD11c⁺ cells, eosinophils, macrophages and lymphocytes were reduced. Decreased FcεRI receptor expression on the recovered platelet population from previously depleted mice, indicates CD11c⁺ cell recruitment and APC activity may be dependent on platelet recognition of allergen (Amison et al., 2018a). Furthermore, allergen sensitization of mice leads to a heightened responsiveness of platelets, suggesting platelets are important in the sensitization of host to allergen and in subsequent allergic inflammatory responses following allergen exposure (Amison et al., 2015).

Platelets from patients with asthma, and mice sensitized to experimental allergen were shown to express FcεRI and the low affinity IgE receptor (FcεRII) (Hasegawa and Matsubara, 2001; Pitchford et al., 2008), which in allergen sensitized mice exposed to allergen, caused platelet migration through the lung tissue via a platelet expressed IgE-/FcεRI-dependent mechanism, indicating direct platelet activation in allergic responses results in platelet recruitment to the lung (Pitchford et al., 2008), and their extravascular presence apparent in

other models of allergic lung inflammation (Chae et al., 2016). The consequences of this accumulation are described below and illustrated in Figure 1.1, where platelets have been shown to contribute to symptoms associated with allergic asthma in animal models of allergic airways disease.

Platelets in bronchoconstriction

Platelets within the lung of asthmatic patients may contribute to the disease progression in a number of ways. Bronchoconstriction causes the narrowing of airways through constriction of airway smooth muscle cells surrounding the airways, which leads to breathlessness, wheezing and coughing. Studies have suggested that platelets are recruited to the airways following exposure to allergen, which might lead to platelet degranulation in the lungs and affect lung function (Pitchford et al., 2008; Page and Pitchford, 2014). This activation and degranulation of platelets can cause the release of stored bronchoactive mediators from granules, such as histamine and adenosine, along with metabolism of arachidonic acid to form 12-Hydroxyeicosatetraenoic acid (12-HETE), serotonin, thromboxane A₂ (TXA₂), PF₄, beta-TG and cytotoxic compounds, which could potentially cause sensory nerve activation and smooth muscle contraction (Page and Pitchford, 2014; Keir et al., 2015). Indeed, platelet depletion in guinea pigs and rabbits prevented bronchoconstriction responses towards allergen, and bronchoactive mediators bombesin, bradykinin and capsaicin, and importantly, had no effect on direct acting spasmogens in the same model, which indicates that platelets can also influence the action of these mediators which did not cause platelet aggregation *in-vitro* (Coyle et al., 1990; Keir et al., 2010b, 2010a, 2015). Furthermore, platelet depletion in a lethal anaphylaxis model of rabbits, led to the survival of rabbits, which had unaltered respiratory frequency (Pinckard et al., 1977). Recent studies have shown that platelet granules are primed with Munc (mammalian homolog of *C. elegans* uncoordinated gene) 13-4 (Munc13-4) before tethering, docking and fusing with the plasma membrane, leading to exocytosis and granular release. The selective deletion of platelet Munc13-4 in OVA-sensitized, OVA-challenged mice, caused decreased airway

hyperresponsiveness, which was measured to increasing doses of methacholine in a murine model of allergic lung inflammation (Cardenas et al., 2018). Therefore, platelets may in some way contribute to the bronchoconstriction that occurs in the early phase response in patients with asthma (Figure 1.1).

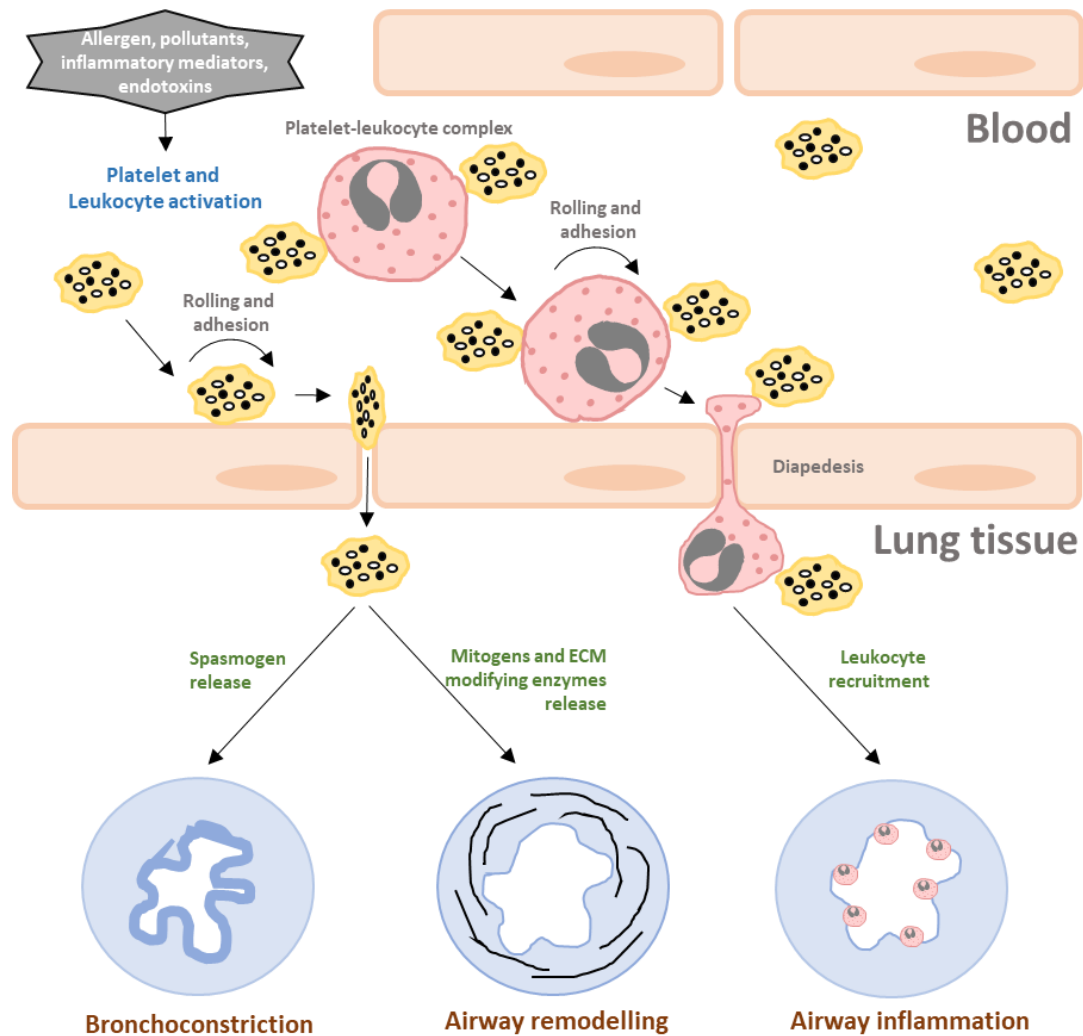


Figure 1.1: Illustration of the effects of platelet activation in allergic asthma

Exposure to noxious stimuli can cause non-thrombotic platelet (yellow cell) activation, which leads to platelet migration and platelet-induced leukocyte (red cell) migration, into lung tissue. Platelets can then effect bronchoconstriction, airway remodelling and airway inflammation, by assisting leukocyte recruitment and through inflammatory mediator release.

Platelets in airway remodelling

Airway remodelling occurs in patients with asthma as a result of chronic airway inflammation leading to a loss of respiratory function and an alteration of the lung architecture,

through collagen deposition, subepithelial fibrosis and goblet cell hyperplasia (Barnes, 2011). Platelets are known to be important for tissue regeneration after injury (Stellos et al., 2010) as they contain many pertinent mitogens responsible for cellular hyperplasia, angiogenesis and nerve regrowth (Page and Pitchford, 2014). Therefore, it has been suggested that platelets might also be involved with exaggerated regenerative responses that are apparent during airway wall remodelling. Indeed, several *in-vitro* studies have shown that platelets stimulate airway smooth muscle cell (ASMC) proliferation, through direct contact of platelet membrane associated factors with human and guinea pig ASMC (Svensson Holm et al., 2008, 2011). Furthermore, platelet depletion in an allergic mouse model caused decreased subepithelial fibre deposition, smooth muscle thickening and epithelial thickening (Pitchford et al., 2004). This effect could be attributed to the fact that platelets produce mitogens such as platelet-derived growth factor, epidermal growth factor, TXA₂, transforming growth factor- β , and vascular endothelial growth factor, which if released from extravascular platelets in the lung, could impact airway remodelling through proliferative effects on cells (Page and Pitchford, 2014). Platelet activation following allergen challenge has been shown to persist and outlast the presence of platelet-leukocyte conjugates in the blood (Kowal et al., 2006). Coupling this persisting activation of platelets with mitogen release, suggests a possible mechanism through which platelets have adverse remodelling effects in the lung (Figure 1.1).

Platelets in airway inflammation

In the chronic phase of the asthmatic response, leukocyte recruitment to the lung is a characteristic feature, leading to excessive airway inflammation and adverse remodelling of the lung architecture. Eosinophil recruitment is typical in such responses, being associated with rhinitis, aspirin-induced exacerbated respiratory disease (AERD) and atopic asthma (Lu et al., 2010; Perić et al., 2011; Laidlaw and Boyce, 2013). Investigations into the roles of platelets in allergic asthma has uncovered that platelets are important in leukocyte (eosinophil) recruitment following allergen challenge, which is discussed below (Idzko et al., 2015).

In patients with asthma, markers of eosinophil and platelet activation are positively associated (Benton et al., 2010). Staining of whole blood leukocytes from patients with mild asthma and AERD patients, showed that between 5-25% of eosinophils had platelets attached (Wardlaw et al., 1992; Johansson and Mosher, 2011; Laidlaw et al., 2012). When platelets were depleted in an allergic guinea pig model of asthma, eosinophil recruitment to the lung was reduced (Lellouch-Tubiana et al., 1988). Likewise, in allergic rabbit and mouse models, platelet depletion caused decreased hyperresponsiveness into the lungs and decreased eosinophil infiltration (Coyle et al., 1990; Pitchford et al., 2003). Therefore, suggesting a possible role of platelets in lung eosinophilia.

The direct physical association of platelets to eosinophils can form through platelet P-selectin and leukocyte PSGL-1 surface expressed adhesion molecule interactions. Indeed, in an *in-vitro* assay investigating platelet and eosinophil adhesion, P-selectin blocking antibodies prevented platelets from ‘rosetting’ around eosinophils (Jawień et al., 2005). Eosinophils have increased surface associated platelets expressing P-selectin in blood taken from non-severe asthma patients, following whole lung antigen challenge (Johansson et al., 2012). Using immunofluorescence microscopy, $\beta 1$ -integrin and P-selectin was colocalized on activated eosinophils (Johansson and Mosher, 2011). Furthermore, $\alpha 4 \beta 1$ -integrin activation was increased on eosinophils, following the addition of P-selectin to whole blood, which also increased eosinophil adhesion *in-vitro* to vascular cell adhesion molecule 1 (Johansson and Mosher, 2011). Blocking antibodies directed against P-selectin caused decreased eosinophil clustering and binding to the endothelium under flow conditions in the blood of patients with asthma (Ulfman et al., 2003), and following asthmatic patients whole lung antigen challenge, eosinophils associated with P-selectin decreased in the circulation (Johansson et al., 2012), suggesting platelet-eosinophil complexes migrate to the lungs. Since platelet P-selectin has been shown to be necessary in neutrophil recruitment to the lung via multiphoton and intravital microscopy (Sreeramkumar et al., 2014; Pan et al., 2015; Riffo-Vasquez et al., 2016;

Zuchtriegel et al., 2016), it is likely P-selectin acts in a similar fashion on eosinophils, leading to eosinophil activation and subsequent migration into the lungs (Pitchford et al., 2005).

Following platelet and eosinophil activation, inflammatory mediators are released through degranulation. Platelets can release PF4 (Hayashi et al., 1994), IL-1 β (Lindemann et al., 2001; Denis et al., 2005), TARC and RANTES (Chihara and Nakajima, 1989; Kameyoshi et al., 1992, 1994; Burgers et al., 1993; Chihara et al., 1994), which can activate eosinophils. Reciprocally, eosinophils can release platelet-activating factor (PAF), eosinophil peroxidase (EPO) and major basic protein (MBP), which can potentially activate platelets (Lee et al., 1984; Rohrbach et al., 1990; Shah et al., 2017). Therefore, platelets can directly and indirectly interact with eosinophils, leading to eosinophil infiltration into the lungs following exposure to allergen. Indeed platelet-leukocyte conjugates formed through platelet P-selectin and leukocyte PSGL-1 adhesion molecule interactions, were shown to assist leukocytes tethering to the endothelium and consequently their diapedeses into inflamed tissue in sensitized mice exposed to allergen (Pitchford et al., 2005). Furthermore, after allergen challenge the infiltration of leukocytes into airways of platelet depleted mice was reduced and could be restored by infusing platelets from allergic mice (Pitchford et al., 2003). Eosinophil recruitment to the lungs in the late phase response of asthma leads to further recruitment of leukocytes through inflammatory mediator release and consequent release of free radicals causing epithelial cell damage and increased mucus production, contributing to airway inflammation.

1.3 Platelet recruitment and migration

1.3.1 Cellular motility and chemotaxis

In order for cells to migrate they must be able to extend protrusions, contract their cytoplasm and then detach from contact sites, which is a process involving actin filament assembly and disassembly causing shape change of the cell (Lauffenburger and Horwitz, 1996). The first step in migration is the polarization of the cell, leading to a clear distinction between the leading edge and trailing edge, often characterised by F-actin redistribution to a particular region (Coates 1992). The leading edge develops lamellipodia and filopodia processes that project around the cell, along with expression of chemosensory signalling receptors (Sullivan et al., 1984). Extension of lamellipodia and filopodia processes are accompanied with actin polymerization, leading to actin filament growth via uncapping existing filaments, severing existing filaments or the formation of new actin filaments (Lauffenburger and Horwitz, 1996). This is a highly regulated process with a fast turn over due to depolymerization of actin. Focal adhesions allow interactions with the extracellular matrix, through integrin clusters tethered to the extracellular matrix, which can form at the leading edge and persist until they reach the trailing edge of the cell (De Pascalis and Etienne-Manneville, 2017). Focal adhesions allow cells to apply traction forces to the extracellular matrix, and myosin intracellular contractile forces can pull the cell body forward. At the trailing edge, adhesions can be disassembled through endocytosis, enzymatic action and proteolytic action (Vicente-Manzanares et al., 2005).

1.3.2 Can platelets migrate?

Platelets have been shown in extravascular compartments and also in fibrous material within the airway luminal edge, in bronchial biopsies of patients with asthma (Metzger et al., 1987; Jeffery et al., 1989). Similarly, platelets have been shown to accumulate into extravascular compartments of the lung in sensitized mice challenged with allergen (Pitchford et al., 2008), in apposition to areas of bronchial smooth muscle and eosinophil infiltration in

guinea pigs (Vargaftig et al., 1982; Lellouch-Tubiana et al., 1988), and separately after LPS exposure (Ortiz-Muñoz et al., 2014) or bacterial infection (Amison et al., 2018b). This phenomenon of platelet accumulation in extravascular compartments has also been recorded in other disease states unrelated to respiratory diseases such as in rheumatoid arthritis and multiple sclerosis, where platelets were found to have migrated extravascularly into inflamed tissue (Boilard et al., 2010; Langer et al., 2012). It has been hypothesised that the presence of platelets in these areas is not due to leakage from blood vessels, but rather through mechanisms of specific recruitment and migration in non-thrombotic conditions (Pitchford et al., 2008).

Platelets roll, firmly adhere and become sessile in aggregatory responses. However, platelets possess the requisite components to migrate and more recently have been reported to undergo actin-cytoskeletal rearrangements, filopodia formation and extend lamellipodia (Bettex-Galland and Luescher, 1959; Pleines et al., 2012; Gaertner et al., 2017), and this has been shown to be dependent on phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) activity (Czapiga et al., 2005; Kraemer et al., 2010). Platelets also contain enzymes such as β -N-acetylhexosaminidase, cathepsin D, cathepsin E and heparinase, which may contribute to movement through connective tissue and the endothelium, as well as matrix metalloproteases, such as MMP2 and MMP9, which may have roles in extracellular matrix degradation of fibrin and collagen (Ciferri et al., 2000; Corry et al., 2002; Falcinelli et al., 2005). Therefore, platelets possess the necessary machinery to undergo migration, which in several studies, has been reported to be directional in response to stimuli or injury and further discussed below.

1.3.3 Platelet chemotaxis

Czapiga and colleagues investigated human platelet chemotaxis via FPR activation by a bacterially derived chemoattractant, N-formyl-methionyl-leucyl phenylalanine (fMLP). FPRs are G-protein coupled receptors (GPCR) that are found in various different cell types and, once activated, can lead to cellular chemotaxis (Panaro et al., 2006). FPR expression and functionality was shown on platelets (Czapiga et al., 2005). The Neuro Probe ChemoTx microplate was used for chemotaxis investigations, which allows cells to pass through a 2 μ m

pore separating a top platelet compartment and bottom chemokine (fMLP) compartment. Following a 2 hour incubation period at 37 °C, platelets in the bottom chamber were counted by light microscopy. Significant platelet chemotaxis to fMLP was observed, revealing the ability of platelets to migrate towards stimuli arising from pathogens. Thrombin stimulation of platelets prior to the addition of platelets to the well, caused elevated responses to fMLP in this assay, suggesting priming of platelets might lead to elevated chemotactic responses (Czapiga et al., 2005).

Following investigative studies that showed platelets are important in airway hyperresponsiveness (AHR) and pulmonary leukocyte recruitment, Pitchford *et al.* investigated platelet migration to the lungs of sensitized mice in response to allergen challenge, and also the ability of platelets from sensitized mice and asthmatic patients, to migrate *in-vitro* toward the specific allergen to which they are sensitised. The Neuro Probe ChemoTx microplate was used, with platelets in the top well and allergen in the bottom well separated by a 3 µm pore sized filter (Boyden, 1962; Valone et al., 1974; Pitchford et al., 2008). Following a 90 minute incubation period, the number of migrated platelets in the bottom well was quantified using light microscopy and expressed as absolute numbers and a 'chemotactic index', defined as 'The average number of migrated platelets in response to a chemotactic stimulus divided by the average number of migrated platelets in response to vehicle' (Pitchford et al., 2008). Platelets from mice and humans significantly migrated toward allergen, compared to vehicle, which interestingly was shown to be a IgE-/FcεRI-dependent mechanism (Pitchford et al., 2008). Although the molecular mechanism governing this migration is not fully understood, GPCRs (including some chemokine receptors) have been shown to interact with FcεRI signalling, which may be involved in cellular chemotaxis (Kuehn and Gilfillan, 2007; Kuehn et al., 2010). The study of platelet migration in direct response to allergen, or as a result of allergen sensitization therefore needs further elucidation.

Human platelet chemotaxis via platelet chemokine receptor activation was first investigated by Kraemer and colleagues. CXCL4 (stromal cell derived factor-1α, SDF-1α), a

CXCR4 receptor ligand, induced platelet chemotaxis which was tested using an improved *in-vitro* assay that more closely mimics the environment *in-vivo* (Kraemer et al., 2010). Light transmission microscopy on fibrinogen-coated chamber slides was used as a platform to investigate platelet chemotaxis to a horizontal gradient. A gel bead containing SDF-1 α was placed in the centre of the well, which slowly released chemokine. Immunofluorescent staining of platelets that surrounded the SDF-1 α gel bead, showed polarized platelets with focal adhesion contacts, indicating platelets were in a migratory state. Platelets within the well were subsequently tracked. Of the motile platelets within the well, significantly more platelets migrated toward SDF-1 α than vehicle. To verify this, the authors also investigated platelet chemotaxis to SDF-1 α using transwell inserts. In the transwell assay platelet chemotaxis was successfully measured towards SDF-1 α , which was importantly inhibited by CXCR4 receptor antagonist AMD3100 (Kraemer et al., 2010). This study demonstrates platelet chemotaxis to an inflammatory chemokine via platelet chemokine receptor activation, although this is yet to be explored in the setting of allergy.

Gaertner and colleagues extensively investigated platelet migration in the context of injury and aggregation, and bacterial infection *in-vivo*. In an *in-vivo* needle and prick model using state of the art imaging techniques, platelets were observed migrating independently of the clot, or bacterial infection, and against the flow of blood along the vessel lumen. These platelets also differed in their mean velocity and migration paths compared to platelets rolling and adhering for clot formation, suggesting a separate phenotype of platelets at the site injury (Gaertner et al., 2017). Platelet spreading and migration was also investigated on a 2D surface *in-vitro*, in which platelets consistently adopted a crescent moon morphology with lamellipodia protrusions at one end and simultaneous retraction at the other end. The migration of platelets at the leading edge was inhibited using cytochalasin D, indicating migration is dependent on actin networks in lamellipodia. The integrin receptor GPIIb/IIIa was found in clusters on lamellipodia, and when this receptor was blocked, platelet migration ceased, demonstrating the importance of GPIIb/IIIa on platelet migration. A fibrinogen surface

induced robust spreading of platelets, but platelet migration only occurred after serum was added. Furthermore, the presence of albumin and calcium in the serum was crucial in platelet shape change and migration (Gaertner et al., 2017). Gaertner and colleagues therefore provide further evidence that platelets have the ability to migrate, which further supports exploring the roles of platelets in allergic inflammatory responses.

1.4 Chemokine receptors

1.4.1 Chemokines and their receptors

Chemokines are low molecular weight soluble chemotactic factors, and are part of the family of cytokines, that regulate leukocyte migration. The chemokine family can be divided into four main groups, which are derived from the location of cysteine residues involved in the formation of disulphide bonds in the primary structure. The largest subfamily, the CC group, consists of 29 ligands in which the first two cystine residues are adjacent. The CXC (21 ligands) and CX3C (1 ligand) groups first two cystine residues are separated by one or three amino acids, respectively. The first and third cystine residues are absent in the XC group, which consists of at least 2 ligands (Murphy et al., 2000; Zlotnik and Yoshie, 2000; Schall and Proudfoot, 2011; Blanchet et al., 2012). The structural homology of chemokines is similar, leading to overlapping functions as they can often display the ability to bind to several distinct chemokine receptor types (Table 2).

Table 2: Human chemokines and their receptors.

CC (blue), CXC (green), XC (yellow) and CX3C (grey) chemokines, their colloquial names and the receptors which they activate are summarised below. Adapted from Schall and Proudfoot, 2011 and Blanchet et al., 2012.

| Name | Colloquial names | Receptors |
|---------------|--------------------------------|--------------------------------------|
| CCL1 | TCA3; I-309 | CCR8 |
| CCL2 | MCP-1; MCAF; JE | CCR2, CCR3, DARC, CCBP2 |
| CCL3 | MIP-1 α ; LD78 α | CCR1, CCR5, CCBP2 |
| CCL3L1 | LD78 β | CCR1, CCR5 |
| CCL3L2 | – | |
| CCL3L3 | LD78 β | CCR1, CCR5 |
| CCL4 | MIP-1 β | CCR5, CCBP2 |
| CCL4L1 | LAG-1 | CCR5, CCBP2 |
| CCL4L2 | LAG-1 | CCR5, CCBP2 |
| CCL5 | RANTES | CCR1, CCR3, CCR5, DARC, CCBP2, CCRL2 |
| CCL7 | MCP-3; MARC | CCR1, CCR2, CCR3, CCR5, DARC, CCBP2 |
| CCL8 | MCP-2 | CCR2, CCR3 |
| CCL11 | Eotaxin | CCR3, CCR5, DARC, D6 |
| CCL13 | MCP-4 | CCR2, CCR3, CCR5, DARC, CCBP2 |

| | | |
|---------------|--|--------------------|
| CCL14 | HCC-1 | CCR1 |
| CCL15 | HCC-2 | CCR1, CCR3, DARC |
| CCL16 | HCC-4; LEC | CCR1 |
| CCL17 | TARC; ABCD-2 | CCR4, DARC, CCBP2 |
| CCL18 | DC-CK1; PARC; AMAC-1 | DARC |
| CCL19 | MIP-3 β ; ELC; Exodus-3 | CCR7, CCRL1, CCRL2 |
| CCL20 | MIP-3 α ; LARC; Exodus-1 | CCR6 |
| CCL21 | 6Ckine; SLC; Exodus-2 | CCR7, CCRL1 |
| CCL22 | MDC; STCP-1; ABCD-1 | CCR4, DARC, CCBP2 |
| CCL23 | CK β 8; MPIF-1 | CCR1, FPR2 |
| CCL24 | Eotaxin-2; MPIF-2 | CCR3 |
| CCL25 | TECK | CCR9, CCRL1 |
| CCL26 | Eotaxin-3; MIP-4 α ; IMAC | CCR3 |
| CCL27 | CTACK; ILC; ESkin | CCR10 |
| CCL28 | MEC | CCR3, CCR10 |
| CXCL1 | GRO- α ; MGSA- α ; MIP-2; KC | CXCR2, DARC |
| CXCL1P | – | |
| CXCL2 | GRO- β ; MGSA- β ; MIP-2 α | CXCR2, DARC |
| CXCL3 | GRO- γ ; MGSA- γ ; MIP-2 β | CXCR2, DARC |
| PF4 | PF4 | CXCR3 |
| PF4V1 | PF4-alt; CXCL4V1 | |
| CXCL5 | ENA-78 | CXCR2, DARC |
| CXCL6 | GCP-2 | CXCR1, CXCR2, DARC |
| CXCL7 | NAP-2; beta-TG; CTAP-III | CXCR2, DARC |
| PPBPL1 | – | |
| IL8 | IL8 | CXCR1, CXCR2, DARC |
| CXCL9 | MIG | CXCR3 |
| CXCL10 | IP10; CRG-2 | CXCR3 |
| CXCL11 | I-TAC | CXCR3, CXCR7, DARC |
| CXCL12 | SDF-1 α | CXCR4, CXCR7 |
| CXCL12 | SDF-1 β | |
| CXCL12 | SDF-1 γ | |
| CXCL13 | BCA1; BLC | CXCR5 |
| CXCL14 | BRAK | |
| CXCL16 | SR-PSOX | CXCR6 |
| CXCL17 | DMC | |
| XCL1 | Lymphotactin; SCM-1 α ; ATAC | XCR1 |
| XCL2 | SCM-1 β | XCR1 |
| CX3CL1 | Fractalkine; Neurotactin; ABCD-3 | CX3CR1 |

Chemokine receptors are part of a bigger superfamily of GPCR, which include receptors for hormones, neurotransmitters and inflammatory mediators (Murdoch and Finn, 2000). There are roughly 20 chemokine receptors (Blanchet et al., 2012). With relevance to this thesis, and focusing on those expressed by platelets (discussed below), the chemokine receptors CCR1, CCR3 and CXCR4 signal through coupling to the Gi family with α_i subunit and CCR4 coupling to Gq family α_q subunit were investigated (Myers et al., 1995; Kowalska et al., 2000; Gillard et al., 2002). CCR1, CCR3 and CXCR4 couple to Gi which inhibits adenylyl cyclase activity and its production of cAMP. cAMP activates protein kinase A (PKA), which downregulates degranulation and can inhibit activation (Murdoch and Finn, 2000). Activation of Gi also causes PI3K activation that activates phospholipase C (PLC), this produces diacyl-glycerol (DAG) and inositol trisphosphate (IP3). CCR4 couples to G α_q which causes PLC activation that catalysis the production of IP3 and DAG (Nardelli et al., 1999). DAG activates protein kinase C (PKC) and the production of IP3 leads to the release of calcium from its intracellular stores. PKC and calcium release can lead to various cellular responses (Brass, 2010). The interactions between chemokines and chemokine receptors are critical in directed migration of cells to sites of injury, signifying their importance in host defence.

1.4.2 Chemokine receptors expressed on platelets

Human platelets have been reported to express the CC chemokine receptors CCR1, CCR3 and CCR4, and CXC chemokine receptor CXCR4 (Clemetson et al., 2000; Kowalska et al., 2000; Abi-Younes et al., 2001). PKC and calcium release are vital to platelet degranulation and are both actions occurring as a result of α_i and α_q signalling pathways (Brass, 2010). PKC phosphorylates actin and along with calcium mobilisation, leading to myosin light chain kinase activation. This results in myosin moving along actin filaments. This can cause trafficking of granules to the cell membrane for contents to be released, increased receptor expression on platelets and platelet shape change (Gear and Polanowska-Grabowska, 2002; Amison, 2014; Golebiewska and Poole, 2014). Through these intracellular pathways,

chemokine receptors can activate platelets, possibly leading to degranulation or migration toward a stimulus.

Functional chemokine ligands; CCL3 (macrophage inflammatory protein 1 α , MIP-1 α) and RANTES bind CCR1; CCL11 (eotaxin) and RANTES bind CCR3 (Pease et al., 1998; Struyf et al., 1998); CCL22 (macrophage derived chemokine, MDC) and TARC bind CCR4 (Imai et al., 1997, 1998); and SDF-1 α binds CXCR4 (D'Apuzzo et al., 1997). MIP-1 α , eotaxin, MDC and SDF-1 α are relatively selective for CCR1, CCR3, CCR4 and CXCR4 receptors, respectively. Platelet chemokine receptors are functionally active, as stimulation with MIP-1 α , eotaxin, MDC and SDF-1 α caused platelet activation as measured via calcium signalling, aggregation, and release of granule content (Clemetson et al., 2000).

1.4.3 Chemokine receptor function on platelets and relevance to allergic inflammation.

CCR1 receptor

Human platelet CCR1 receptor activation via MIP-1 α caused increased platelet cytoplasmic calcium levels, proving the functional activity of the receptor (Clemetson et al., 2000). Other parameters of platelet activation have not been investigated with the CCR1 specific chemokine MIP-1 α , however stimulating platelets with RANTES, which also has activity on CCR3, caused a low level aggregatory response as measured with an aggregometer (Clemetson et al., 2000).

The CCR1 receptor is expressed on a number of different cell types and has been shown to induce chemotaxis on cells such as eosinophils and mast cells (Phillips et al., 2003; Toda et al., 2004; Solari and Pease, 2015). In patients with asthma, elevated levels of both MIP-1 α and RANTES were detected in the bronchoalveolar lavage fluid before and after allergen challenge (Cruikshank et al., 1995; Alam et al., 1996), suggesting a role of the CCR1 receptor in allergic inflammatory responses. Following Fc ϵ RI activation on mast cells, CCR1 receptor is upregulated, which enhances Fc ϵ RI mediated calcium generation and degranulation (Kuehn and Gilfillan, 2007). The CCR1 receptor has been shown to co-localize on mast cells with

FcεRI receptors, and when both receptors were activated, mast cell chemotaxis to CCL2 (monocyte chemoattractant protein 1, MCP-1) decreased and degranulation increased (Fifadara et al., 2010), indicating the CCR1 receptor is involved in allergic inflammatory responses. Therefore, investigating the roles of the CCR1 receptor on platelet migration following allergen sensitization, would be of interest.

CCR3 receptor

Human platelet CCR3 receptor expression has been demonstrated by flow cytometry of washed platelets and polymerase chain reaction amplification of platelet mRNA (Clemetson et al., 2000). The functionality of the platelet CCR3 receptor was confirmed via increased intracellular calcium concentrations following eotaxin administration, and caused low level aggregatory responses (Clemetson et al., 2000). CCR3 is a promiscuous receptor in that it binds several chemokine ligands, including each of the eotaxin family (eotaxin-1, eotaxin-2 and eotaxin-3) (Blanchet et al., 2012). The receptor is also expressed on a number of different cell types including eosinophils, T cells, basophils and mast cells (Solari and Pease, 2015).

In allergic lungs of asthmatic patients, increased expression of ligands that bind to CCR3: eotaxin-1, eotaxin-2 and RANTES were measured, moreover, elevated eotaxin-1 levels have been shown in acute compared with stable asthmatic patients (Ying et al., 1997, 1999; Lilly et al., 1999). Eotaxin-1 and eotaxin-2 levels were elevated in sputum samples of patients with asthma (Yamada et al., 2000). Furthermore, CCR3 receptor expression levels have also been shown to be elevated on leukocytes in allergic conditions, suggesting the CCR3 receptor has roles in allergic disease (Francis et al., 2007).

The CCR3 receptor is crucial in eosinophil recruitment to the lungs (Humbles et al., 2002; Fulkerson et al., 2006). Indeed in CCR3 deficient mice, an airway allergen challenge was unable to induce eosinophil migration (Humbles et al., 2002). Similarly, eotaxin-1 and eotaxin-2 deficient mice had reduced pulmonary tissue eosinophilia (Pope et al., 2005). Thus, CCR3 receptor expression and activity has been shown to increase in sensitized states,

furthermore the receptor has demonstrated key functional roles in eosinophil migration into inflamed tissue, suggesting platelet CCR3 receptor may be important in platelet recruitment and migration to sites of allergic inflammation.

CCR4 receptor

Stimulating human platelets with MDC, a CCR4 receptor chemokine, caused platelet activation as determined by calcium signalling, granule content release, platelet shape change and low levels of platelet aggregation (Clemetson et al., 2000; Kowalska et al., 2000; Abi-Younes et al., 2001; Gear et al., 2001). The CCR4 receptor is expressed on dendritic cells, basophils, natural killer cells and T cells, and can be activated by MDC and TARC (Solari and Pease, 2015). The ability of CCR4 receptor stimulation to induce platelet chemotaxis is yet to be investigated. Both MDC and TARC are upregulated in the lungs following allergen challenge in asthmatic patients (Bochner et al., 2003; Pilette et al., 2004). CCR4 receptor expression is upregulated in Th2 cells, which are found in bronchoalveolar lavage fluid of asthmatic patients, likely induced by MDC and TARC release from dendritic cells (Bonecchi et al., 1998; Sallusto et al., 1998; Panina-Bordignon et al., 2001; Morgan et al., 2005). Likewise, T cells isolated from asthmatic patients had elevated expression of the CCR4 receptor (Vijayanand et al., 2010). In CCR4 deficient mice and following antibody neutralisation of MDC and TARC, there was reduced leukocyte recruitment to the lung following allergen challenge (Lloyd et al., 2000; Kawasaki et al., 2001; Mikhak et al., 2009).

The evidence suggests a role for the CCR4 receptor on cellular recruitment and migration in allergic asthma, together with the clear activation of platelets *in-vitro* via stimulation with MDC, the data suggests a role for the CCR4 receptor in allergic inflammatory responses of platelets which is worth investigating (Kowalska et al., 2000; Abi-Younes et al., 2001).

CXCR4

The CXCR4 receptor has been reported to bind SDF-1 α and macrophage migration inhibitory factor (MIF), and is extensively expressed on different cell types (Blanchet et al., 2012). There are several reports of platelet CXCR4 receptor activation following SDF-1 α treatment, including measurements of intracellular calcium concentrations, P-selectin expression, platelet shape change and a low level aggregatory responses (Clemetson et al., 2000; Kowalska et al., 2000; Gear et al., 2001; Kraemer et al., 2010). Platelet chemotaxis was successfully measured towards SDF-1 α , demonstrating the ability of platelets to migrate through chemokine receptor activation (Kraemer et al., 2010). However, the involvement of CXCR4 receptor on platelet activation has not been studied in the context of allergic inflammation.

In patients with asthma, eosinophils from the bronchoalveolar lavage fluid have increased expression of the CXCR4 receptor (Nagase et al., 2000). AMD3100 (CXCR4 receptor antagonist) administration in an allergic murine model decreased inflammation and AHR, following allergen challenge (Lukacs et al., 2002). When neutralizing antibodies directed against SDF-1 α were administered in OVA-sensitized mice challenged with OVA, lung eosinophilia and AHR were reduced (Gonzalo et al., 2000). Furthermore, IL-4 increased CXCR4 receptor expression on Th2 cells (Jourdan et al., 1998). The activation of CXCR4 receptor in allergic inflammation affects multiple cells, therefore the effect of allergen sensitization on CXCR4 receptor induced platelet responses is of interest.

1.5 Aims and objectives

An overall hypothesis to this project is that platelets express chemokine receptors that are functionally relevant for platelet chemotaxis in the context of platelet recruitment and migration during allergic inflammation.

Specific objectives are described below:

- To quantify platelet recruitment and migration to different lung compartments from healthy, non-fatal asthmatic and fatal asthmatic patients.
- To create and characterise *in-vitro* platelet chemotaxis models.
- To create a model for investigating platelet recruitment and migration in response to allergen challenge *in-vivo*.
- To explore the roles of platelet chemokine receptors
 - To investigate the expression of platelet chemokine receptors and the effects of allergen sensitization on their expression.
 - To investigate if chemokine receptor activation can induce platelet chemotaxis.
 - To investigate the effects of allergen sensitization on platelet chemotaxis towards chemokines.
 - To explore the roles of platelet chemokine receptors on platelet recruitment and migration in an allergic inflammatory model.

Chapter 2 Materials/Methods

2.1 Materials

| | |
|--|---------------------------|
| Acid alcohol differentiation solution | Sigma Aldrich (UK) |
| Acid citrate dextrose (ACD) | Sigma Aldrich (UK) |
| Adenosine diphosphate (ADP) | Sigma Aldrich (UK) |
| Albumin from chicken egg white (ovalbumin) Grade V | Sigma Aldrich (UK) |
| Aluminium hydroxide | EMS Pharma (Brazil) |
| AMD3100 (CXCR4 antagonist) | Tocris Biosciences (UK) |
| Bovine serum albumin (BSA) | Sigma Aldrich (UK) |
| C-021 (CCR4 antagonist) | Tocris Biosciences (UK) |
| CaCl ₂ | Sigma Aldrich (UK) |
| Cell Lysis Buffer | New England Biolabs (UK) |
| D. Pteronyssinus crushed whole bodies. Mite, dust. | Stallergenes Greer (USA) |
| DAB Peroxidase (HRP) Substrate Kit | Sigma Aldrich (UK) |
| Depex (DPX) mountant | Sigma Aldrich (UK) |
| Dextrose | Sigma Aldrich (UK) |
| Dimethyl sulfoxide (DMSO) | Sigma Aldrich (UK) |
| Evans Blue Dye $\geq 75\%$ | Sigma Aldrich (UK) |
| Fibrinogen | Sigma Aldrich (UK) |
| Fibronectin | Sigma Aldrich (UK) |
| Flow-count Fluorospheres | Beckman Coulter Inc (UK) |
| Glucose | Fisher (UK) |
| Goat anti-rabbit IgG (biotinylated) | Vector Laboratories (USA) |
| Goat anti-rabbit IgG, HRP-linked antibody | New England Biolabs (UK) |

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| Haematoxylin | Sigma Aldrich (UK) |
| Hamster anti-mouse CD49b phycoerythrin | BD Biosciences (UK) |
| HEPES (4-(2-hydroxyethyl)-1piperazineethanesulfonic acid) | Sigma Aldrich (UK) |
| Hydrogen peroxide | Sigma Aldrich (UK) |
| Industrial ethanol $\geq 99.8\%$ | Sigma Aldrich (UK) |
| Isocare isoflurane anaesthetic | Baxter International, (USA) |
| KCl | Sigma Aldrich (UK) |
| KH ₂ PO ₄ | Sigma Aldrich (UK) |
| Kwik Diff differential stain | ThermoFisher Scientific (UK) |
| Li ₂ CO ₃ | Sigma Aldrich (UK) |
| MgCl ₂ | VWR International (UK) |
| MgCl ₂ | Sigma Aldrich (UK) |
| MgSO ₄ | Sigma Aldrich (UK) |
| Mouse IgG1 κ Isotype Control phycoerythrin | BD Biosciences (UK) |
| Mouse IgG2b κ Isotype Control fluorescein isothiocyanate | BD Biosciences (UK) |
| Na ₂ HPO ₄ | VWR International (UK) |
| NaCl | Fisher (UK) |
| NaHCO ₃ | Fisher (UK) |
| Formylmethionyl-leucyl-phenylalanine (fMLP) | Sigma Aldrich (UK) |
| NH ₄ Cl | Sigma Aldrich (UK) |
| NovaRED Peroxidase (HRP) Substrate | Vector Laboratories (USA) |
| NuPAGE™ 10% Bis-Tris Protein Gels, 12-well | ThermoFisher Scientific (UK) |
| NuPAGE™ Antioxidant | ThermoFisher Scientific (UK) |
| NuPAGE™ LDS Sample Buffer | ThermoFisher Scientific (UK) |

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|--|------------------------------|
| NuPAGE™ MOPS SDS Running Buffer | ThermoFisher Scientific (UK) |
| NuPAGE™ Sample Reducing Agent | ThermoFisher Scientific (UK) |
| NuPAGE™ Transfer Buffer | ThermoFisher Scientific (UK) |
| Paraformaldehyde | Sigma Aldrich (UK) |
| Phosphatase Inhibitor Cocktail 2 | Sigma Aldrich (UK) |
| Phosphatase Inhibitor Cocktail 3 | Sigma Aldrich (UK) |
| Phosphate buffered saline (PBS) tablets | Oxoid (UK) |
| Pierce™ BCA Protein Assay Kit | ThermoFisher Scientific (UK) |
| Pierce™ ECL Plus Western Blotting Substrate | ThermoFisher Scientific (UK) |
| Ponceau S solution | Sigma Aldrich (UK) |
| Precision Plus Protein Standard | Bio-Rad Laboratories (UK) |
| Prostaglandin E1 (PGE ₁) | Sigma Aldrich (UK) |
| Protease Inhibitor Cocktail | Sigma Aldrich (UK) |
| Rabbit anti-human CD42b antibody [SP219] | Abcam (UK) |
| Rabbit anti-mouse CCR1 antibody | Novus Biologicals (UK) |
| Rabbit anti-mouse CCR3 antibody | Novus Biologicals (UK) |
| Rabbit anti-mouse CCR4 antibody | Novus Biologicals (UK) |
| Rabbit anti-mouse CXCR4 antibody | Novus Biologicals (UK) |
| Rabbit anti-mouse β -actin antibody [Poly6221] | BioLegend (UK) |
| Rat anti-mouse CCR1 phycoerythrin antibody [643854] | R&D Systems (UK) |
| Rat anti-mouse CCR3 phycoerythrin antibody [83101] | R&D Systems (UK) |
| Rat anti-mouse CCR4 phycoerythrin antibody [2G12] | BioLegend (UK) |
| Rat anti-mouse CD41 fluorescein isothiocyanate | BD Biosciences (UK) |
| Rat anti-mouse CXCR4 phycoerythrin antibody [247506] | R&D Systems (UK) |

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| Recombinant human eotaxin/CCL11 | PeptoTech (UK) |
| Recombinant Murine MDC/CCL22 | PeptoTech (UK) |
| Recombinant Murine MIP-1 α /CCL3 | PeptoTech (UK) |
| Recombinant Murine SDF-1 α /CXCL12 | PeptoTech (UK) |
| Restore™ Western Blot Stripping Buffer | ThermoFisher Scientific (UK) |
| Roswell Park Memorial Institute (RPMI) 1640 Media | Invitrogen Life Sciences (UK) |
| Saline | Baxter Healthcare Ltd (UK) |
| SB 328437 (CCR3 antagonist) | Sigma Aldrich (UK) |
| Stromatol | Mascia Brunelli SpA (Italy) |
| Tris Base (2-Amino-2-(hydroxymethyl)-1,3-propanediol) | Sigma Aldrich (UK) |
| Tri-sodium citrate | Fisher Scientific (UK) |
| Türk's solution | Merck Millipore, (USA) |
| Tween™ 20 | Fisher Scientific (UK) |
| Urethane | Sigma Aldrich (UK) |
| Vectastain Elite ABC HRP kit | Vector Laboratories (USA) |
| Vitronectin | Sigma Aldrich (UK) |
| Weigert's iron hematoxylin kit | EMD Millipore (USA) |

2.2 Buffer preparations

Phosphate buffered saline (PBS) was prepared for flow cytometry, immunohistochemistry, allergen-sensitization and challenge experiments. PBS tablets were dissolved in distilled water, producing a solution of 3 mM KCl, 1 mM KH_2PO_4 , 8 mM Na_2HPO_4 and 160 mM NaCl at pH7.3.

For intravital microscopy experiments, Tyrode's buffer was used, which contained 133.9 mM NaCl, 2.9 mM KCl, 0.86 mM Na_2HPO_4 , 1 mM MgCl_2 , 5.5 mM dextrose and 20 mM HEPES in distilled water, at pH7.3 and heated to 37°C.

Modified Tyrode's buffer was prepared for mouse platelet isolation and chemotaxis assays. Modified Tyrode's buffer consisted of 133.9 mM NaCl, 2.9 mM KCl, 0.86 mM Na_2HPO_4 , 11.9 mM NaHCO_3 , 20 mM HEPES, 1 mM MgCl_2 , 4 mM glucose and 10 g/L BSA, dissolved in distilled water and adjusted to pH7.3.

Red cell lysis buffer used during platelet isolation for microfluidic chemotaxis assay experiments, contained 654 mM NH_4Cl , 24.8 mM KCl, 3.9 mM Na_2HPO_4 , 0.9 mM KH_2PO_4 , 27.8 mM glucose, 20.7 mM MgCl_2 , 5.7 mM MgSO_4 , 30.6 mM CaCl_2 and 267.8 mM NaHCO_3 , dissolved in distilled water.

Sodium citrate buffer that was prepared for antigen retrieval steps in immunohistochemistry, contained 113.9 mM tri-sodium citrate and 0.05% Tween 20, dissolved in distilled water at pH6.0.

Tris-buffered saline with Tween 20 (TBST) consisted of 20 mM Tris, 150 mM NaCl and 0.1% Tween 20 at pH7.5. TBST and all other buffers used in western blot analysis, were dissolved in ultrapure water.

2.3 *In vivo studies*

2.3.1 *Animals*

Animal experiments were conducted in accordance with the Animals (Scientific Procedures) Act 1986 and amended regulations of 2012, with approval from the local Animal Welfare and Ethics Committees at King's College London and the Babraham Institute, Cambridge. Male and Female BALB/c inbred mice (20-25 g) were procured from Envigo and Harlan Laboratories (UK). Male and female inbred C57BL/6 wild type (WT) mice (20-25g) were bred in pathogen-free facilities at the Babraham Institute. All mice were between 6-10 weeks of age and given a 7 day acclimatisation period on delivery. Mice were housed in cages with environmental enrichment (Datesand, UK), autoclaved aspen woodchip bedding, had access to water and an RM1(E) diet (Special Diets Services, UK). Temperature was regulated to 19-22°C, at a relative humidity of 45-65% and a 12:12 hour light:dark cycle.

2.3.2 *Mouse sensitization and challenge to OVA*

Mice were sensitized to OVA (30 µg in 0.4 ml PBS with 0.1 M Al₂(OH)₃ adjuvant) intraperitoneally (i.p.) on days 0, 4 and 11. Sham-sensitized mice were administered 0.4 ml PBS with 0.1 M Al₂(OH)₃ i.p. For comparisons between sham-sensitization and OVA-sensitization, and the influence of allergen exposure (e.g. chemotaxis studies, and assessment of chemokine receptor expression), mice were either culled on day 14 and platelets harvested via blood collection using cardiac puncture; or mice were exposed to aerosolised OVA (30

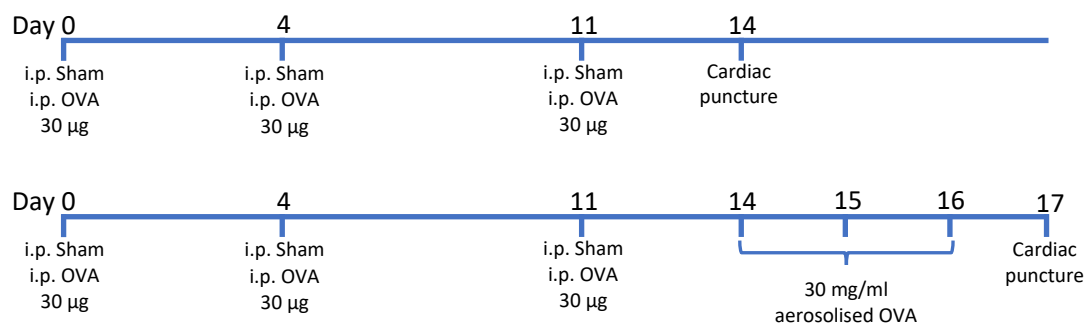


Figure 2.1: Mouse OVA-sensitization and challenge protocol.

mg/ml) for 30 minutes on days 14, 15, 16 and culled on day 17 before platelets were harvested (Amison et al., 2015) (Figure 2.1).

2.3.3 Mouse sensitization to HDM

Whole bodies of the house dust mite *Dermatophagoides Pteronyssinus* were crushed by mortar and pestle and prepared by Stallergenes Greer (USA). *Dermatophagoides Pteronyssinus* extract (HDM) peptidase 1 enzyme allergen, Der p 1, was quantified as 54 µg Der p 1 per 1 mg of total protein. Mice were anaesthetised with isoflurane before sensitization with 25 µg/25 µl HDM or 25 µl saline i.n. on days 0, 1, 2, 3, 4, 7, 8, 9, 10 and 11 (Gregory et al., 2009) (Figure 2.2).

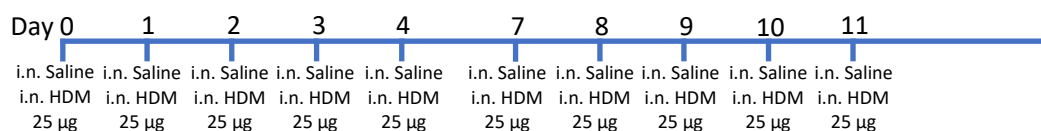


Figure 2.2: Mouse HDM-sensitization protocol.

2.3.4 HDM challenge and bronchoalveolar lavage fluid collection

Following the allergen sensitization protocol in section 2.3.3, on day 13, 25 µg/25 µl HDM or 100 µg/100 µl HDM was administered i.n. (Figure 2.3) with corresponding sham-sensitized groups receiving 25 µl or 100 µl of saline, respectively. 24 hours later mice were terminally anaesthetised with 2.1 g/kg urethane i.p. and the trachea cannulated. 0.5 ml of saline was injected into the cannulated trachea and drawn back into the syringe for collection. This was repeated three times, given approximately 1.5 ml bronchoalveolar lavage (BAL) fluid, which was then placed on ice.

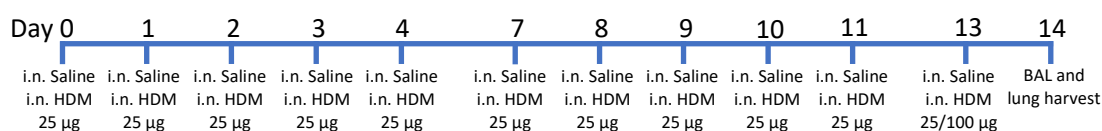


Figure 2.3: Mouse HDM-sensitization and i.n. HDM challenge protocol.

2.3.5 Total cell counts and differential cell quantification

BAL fluid was mixed 1:1 with Türk's solution and the total leukocyte cell number quantified using a Neubauer improved haemocytometer under a phase contrast light microscope (Zeiss Axiovert) with a 20x objective lens.

For differential cell quantification, 100 µl BAL fluid was centrifuged using a Shandon Cytospin 3 on Superfrost Plus slides at 1000rpm for 1 minute. The slides were then stained using Kwik Diff differential stain before cover slipping with DPX mounting medium. The percentage of neutrophils, macrophages, lymphocytes and eosinophils was calculated by counting 200 cells from four random fields of view, using a phase contrast light microscope (Zeiss Axiovert) with a 40x objective lens. The total number of each cell type was then calculated from the total cell counts.

2.3.6 Lung harvesting for histology and immunohistochemistry

Anaesthetised mice, tracheas were cannulated and lungs inflated with 0.5 ml of 3.7% paraformaldehyde (PFA). The lungs were dissected and stored in 3.7% PFA, before tissue processing and wax embedding (section 2.6.1).

2.3.7 HDM-sensitized mice HDM challenge following an extended recovery period

Due to elevated numbers of leukocytes in the lungs of sham-sensitized mice following a day 13 challenge, an additional 7 day recovery period was used (Figure 2.4). Following the allergen-sensitization protocol in section 2.3.3, mice were challenged on day 20 with HDM (100 µg/ 100 µl i.n.). 24 hours later, the lungs were harvested for histology and immunohistochemistry (section 2.3.6).

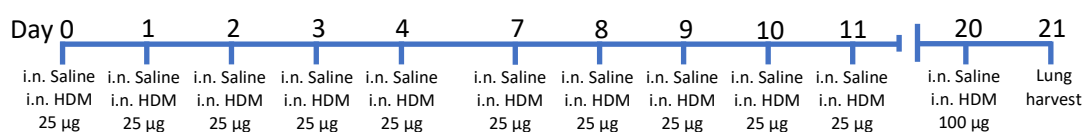


Figure 2.4: Mouse HDM-sensitization and i.n. HDM challenge with extended protocol.

2.3.8 Intravital microscopy of mouse cremaster muscle

The cremaster muscle is a thin piece of muscle tissue surrounding the testis, which can be externalised and viewed, to allow clear visualisation of the vasculature. In order to visualise platelets in cremaster muscle preparations, hamster anti-mouse CD49b (phycoerythrin) PE conjugated antibody (1.6 $\mu\text{g}/0.1\text{ mL}$) was injected intravenously (i.v.) 1 hour before cremaster muscle dissection. Mice were terminally anaesthetised with urethane (2.1 g/kg i.p.) and had their temperature regulated with a heated mat. A small incision on the right ventral scrotal sack allowed extrusion of a single testis from the scrotal sack. The inferior part of the cremaster muscle surrounding the testis was pinned to the viewing window and an incision was made along the medial side of the cremaster muscle from the inferior pinned section. The cremaster muscle was then folded over laterally and pinned to the viewing window (Figure 2.5).

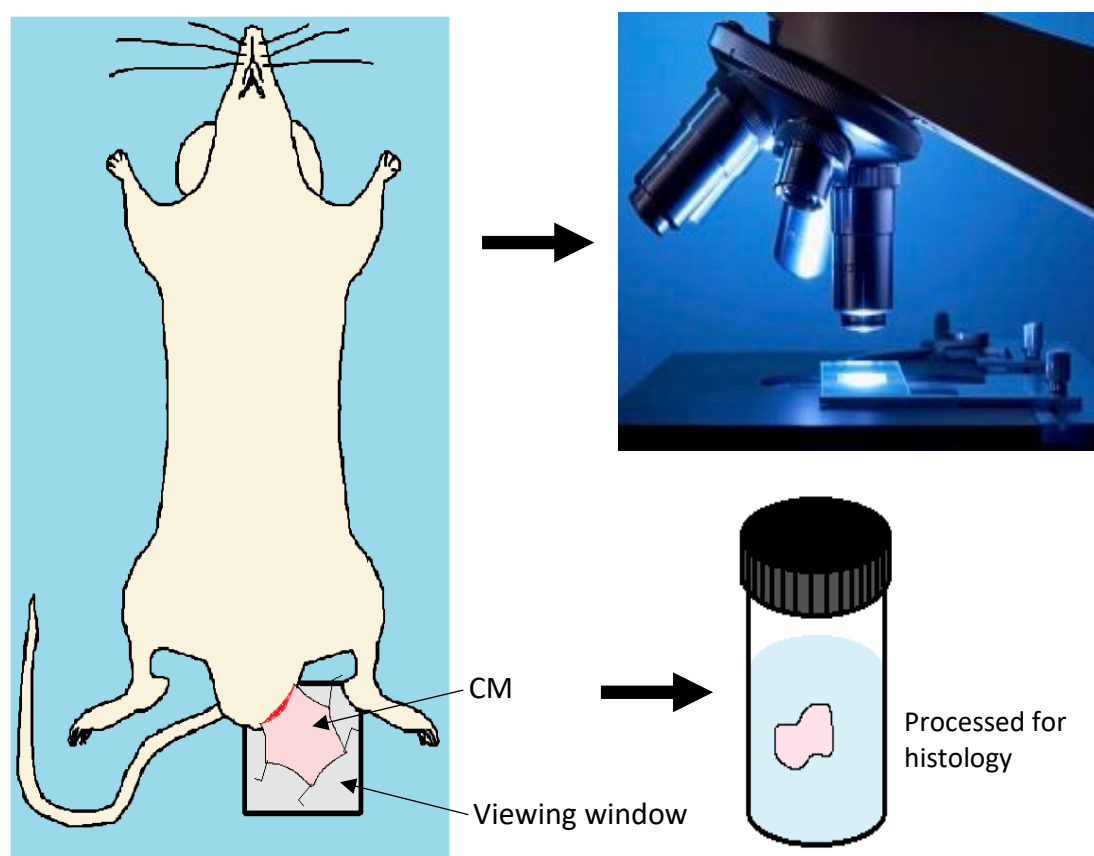


Figure 2.5: Illustration of mouse cremaster muscle preparation for intravital microscopy.

The cremaster muscle was mounted under a Zeiss Axioskop 2 inverted reflective fluorescence microscope with a digital CMOS ORCA-Flash 2.8 camera (Hamamatsu) and a water immersed 63x objective lens. The scale and area of cremaster muscle viewed was determined using a micro-ruler. The exposed tissue was superfused with 37°C Tyrode's buffer using a Watson-Marlow pump at 12 ml/min and excess Tyrode's buffer suctioned from the viewing window. Under 580 nm fluorescent light and a dimmed bright field light, both the platelets and the architecture of the post capillary venules could be observed. Post capillary venules were distinguished from arterioles by following the flow of blood to a convergence point, indicating the vessels under view were part of the venous blood circulation.

A minimum of 3 different post capillary venules from each mouse were recorded for 10 seconds. The number of leukocytes present in extravascular compartments of cremaster muscle were counted and expressed per area of cremaster muscle. Platelet adhesion events were classified as instances where a fluorescent platelet would remain bound to the same section of endothelium for the duration of a 10 second video and expressed per area of cremaster muscle. Platelet rolling events were classified as instances where a fluorescent platelet would slow down, stop or move off the endothelium during the 10 second video.

2.3.9 Establishing an allergic inflammatory response in the cremaster muscle

Sham-sensitized and OVA-sensitized mice (section 2.3.2) were challenged with 4x the sensitization dose, to establish whether an allergic inflammatory response could be generated. On day 14, OVA (150 µg/100 µl) was administered subcutaneously (s.c.) on the scrotum and 4 hours later the cremaster muscle visualized. Aluminium hydroxide is used as an adjuvant in the OVA-sensitization protocol which tended to accumulate within the testis of sham-sensitized and OVA-sensitized mice, causing a severe inflammatory response. Consequently HDM-sensitization of mice was explored to investigate allergic inflammatory responses in the cremaster muscle where there was no requirement for an adjuvant.

Sham-sensitized and HDM-sensitized mice (section 2.3.3) were challenged on day 13 with saline or HDM (100 µg/100 µl s.c.) on the scrotum. 24 hours later the cremaster muscle was dissected and visualized (Figure 2.6).



Figure 2.6: Mouse HDM-sensitization and HDM s.c. challenge protocol.

2.3.10 Cremaster muscle harvesting for histology and immunohistochemistry

Following intravital microscopy video capture, the cremaster muscle was dissected off the testis and placed in 3.7% PFA. 24 hours later, the cremaster muscle was processed and wax embedded to allow for histological and immunochemical staining (section 2.6.1).

2.3.11 Chemokine receptor antagonist treatment

The CCR3 receptor antagonist SB328437 (N-(1-Naphthalenylcarbonyl)-4-nitro-L-phenylalanine methyl ester) and the CCR4 receptor antagonist C-021 (2-[1,4'-Bipiperidin]-1'-yl-N-cycloheptyl-6,7-dimethoxy-4-quinazolinamine dihydrochloride) were both prepared in DMSO, while the CXCR4 receptor antagonist AMD3100 (1,1'-[1,4-Phenylenebis(methylene)]bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride) was prepared in saline. SB328437, C-021 and AMD3100 are highly specific antagonists of CCR3, CCR4 and CXCR4 receptors respectively (White et al., 2000; Hatse et al., 2002; Yokoyama et al., 2009). Doses of 30 mg/kg SB328437, 30 mg/kg C-021 and 10 mg/kg AMD3100, were determined based on previous literature where these antagonists showed efficacy *in-vivo* (Mori et al., 2007; Yokoyama et al., 2009; Li et al., 2011; Yu and Hales, 2011; Chen et al., 2016). Chemokine receptor antagonists were administered i.p. 30 minutes before allergen challenge, with control groups and the AMD3100 group, receiving equivalent amounts of DMSO. An allergen challenge with HDM (100 µg/100 µl) was administered s.c. to the

scrotum in intravital microscopy preparations, and an allergen challenge with HDM (100 µg/100 µl) was administered i.n. in allergic lung studies.

2.3.12 Exogenous eotaxin administration to the scrotum for intravital microscopy

The effects of eotaxin administration to the scrotum of HDM-sensitized mice, on platelet intravascular activity was explored using intravital microscopy of mouse cremaster muscle. Based on previous literature from our group, mice received eotaxin (0.1 µg s.c.) in the scrotum on day 14 (Riffo-Vasquez et al., 2012). After 4 hours, the cremaster muscle was prepared for intravital microscopy as described above.

2.3.13 Bleeding time measurements following injury

Mice sensitized to HDM as previously described in section 2.3.3, were administered chemokine receptor antagonists before an allergen challenge with HDM (100 µg/100 µl i.n.) (section 2.3.9) and subsequently had their bleeding times measured 24 hours later. Mice were anaesthetised with isoflurane prior to resecting 2 mm from the distal end of their tail with a sharp blade. The tail was immediately immersed in 37°C saline and the time taken for cessation of bleeding recorded (Greene et al., 2010).

2.3.14 Evans blue allergic skin test

To show evidence of systemic sensitization of mice, skin testing with the allergen was carried out in animals receiving Evans blue dye to assess plasma protein extravasation. Following HDM-sensitization as described in section 2.3.3 above, on day 13 the dorsum fur of mice was shaved off. On day 14, 30 minutes before allergen challenge in skin, mice were administered Evans blue dye (0.5% w/v 0.1 ml i.v.). Sham-sensitized and HDM-sensitized mice were administered HDM intradermally (25 µg/25 µl i.d.), and the equivalent volume of saline at two separate injection sites on their dorsum. Visual assessments of plasma protein extravasation around the injection site were made from pictures captured on an iPhone X (Apple) of the extravascular blue dye.

2.4 *In vitro* chemotaxis assays

2.4.1 *Mouse washed platelet preparation*

Mice were terminally anaesthetised with urethane (2.1 g/kg i.p.), and 0.6-0.9 ml of blood was collected via cardiac puncture, into 0.1 ml ACD. Blood was then centrifuged at 300g for 3 minutes and the platelet rich plasma (PRP) supernatant was collected. The blood was centrifuged again at 300g for 3 minutes, in order to collect the remaining PRP. PGE₁ (60 nM final concentration) was added to PRP and then centrifuged at 800g for 6 minutes and platelet poor plasma (PPP) aspirated off. The platelet pellet was re-suspended in 200 µl of modified Tyrode's buffer. 2 µl of washed platelets was added 1:100 to Stromatol platelet counting solution, and the number of platelets determined using a haemocytometer and a phase contrast light microscope (Zeiss Axiovert) with 40x objective lens.

Centrifuging platelets at high speeds leads to platelet activation and aggregation, therefore PGE₁ was added to PRP to minimise platelet activity. PGE₁ stimulates adenylyl cyclase activity and increases cyclic AMP concentrations, which inhibits platelet aggregation induced by P2Y₁ receptor activation.

For platelet preparations used in Ibidi µ-Slide VI^{0.4} microfluidic chamber (microfluidic chemotaxis assay) experiments, an additional step was used after centrifugation at 800g for 6 minutes. The platelet pellet was re-suspended in 200 µl red cell lysis buffer and incubated for 4 minutes before adding 1 ml of modified Tyrode's buffer. PGE₁ (60 nM) was again added to the platelets and centrifuged at 800g for 6 minutes, with the remainder of the protocol followed.

2.4.2 *Microplate chemotaxis assay for the evaluation of mouse platelet chemotaxis towards different chemokines*

Washed platelets were prepared as described above (section 2.4.1) from sham-sensitized or OVA-sensitized mice (section 2.3.2) and diluted to 1×10^8 platelets/ml in modified Tyrode's buffer. 96-well ChemoTx® System (Neuro Probe, USA) microplates, with

2 μm pore size, were blocked with 30 μl /well modified Tyrode's buffer for 1 hour. Chemokines: MIP-1 α , eotaxin, MDC and SDF-1 α were prepared at 10 nM and 100 nM, with a separate control for each chemokine to minimize cross plate variability. 31.5 μl /well chemokine was added and the filter placed on top.

Previous data from our laboratory reported that platelet priming via the P2Y₁ receptor causes an increased response of platelets to chemoattractants (Amison et al., 2015, 2018a). Therefore platelets were stimulated with a sub-aggregatory concentration of ADP, to provide a more robust response in chemotaxis assays.

Washed platelets were treated with ADP (0.1 μM) for 10 minutes, before adding 24 μl washed platelets on the filter (Figure 2.7). The chambers were then incubated at 37°C for 120 minutes. The filter was then removed and bottom well contents added 1:1 to Stromatol. Platelets were enumerated on a haemocytometer using a phase contrast light microscope (Zeiss Axiovert) with a 40x objective lens.

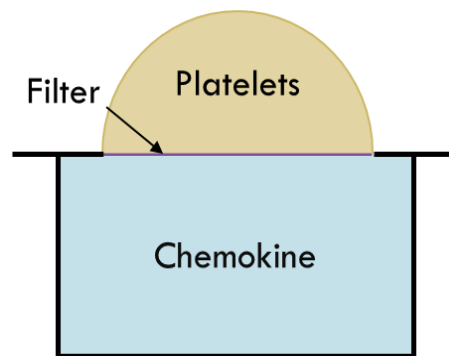


Figure 2.7: Microplate chemotaxis assay arrangement.

2.4.3 Characterisation experiments of microfluidic chemotaxis assay to investigate platelet chemotaxis

Washed platelets were prepared from WT mice (section 2.4.1) and diluted to 1×10^7 platelets/ml. Washed platelets were then treated with 2 mM CaCl_2 30 minutes before application to the microchamber. The Ibidi $\mu\text{-Slide VI}^{0.4}$ microfluidic chamber (Ibidi,

Germany) was incubated with different coating solutions (e.g. 10% mouse serum (commercially unavailable), 10 $\mu\text{g/ml}$ vitronectin and 10 $\mu\text{g/ml}$ fibrinogen) for 30 minutes before rinsing through with modified Tyrode's buffer. 50 μl of platelet suspension was added to one end of the well, then 50 μl was drawn through the other end, causing platelets to be pulled into the centre of the chamber, where they were allowed to settle on the well (time dependency was investigated as a variable). 1.14×10^8 beads/ml (Bangs Laboratories, UK) were added to modified Tyrode's buffer control and to chemokines MDC, eotaxin and SDF-1 α , which were prepared at 1, 10, 100 and 1000 nM. Excess platelets from each side well were drained, leaving approximately 30 μl of platelets in the well. 5 μl of chemokine or control were added directly into the well at one end and 5 μl removed from the other end (Figure 2.8). Before starting recordings, slides were given time for the beads to settle and for a chemokine gradient to establish (time dependency was investigated as a variable).

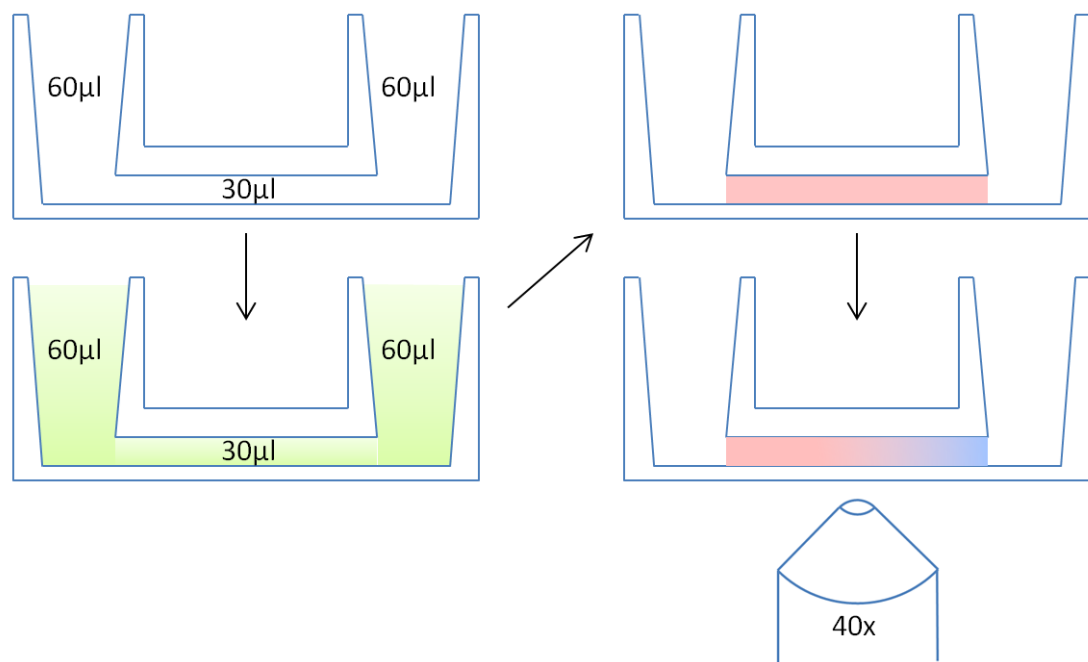


Figure 2.8: Diagrammatic representation of microfluidic chemotaxis assay.

Slides were filled with a coating solution (green), washed through with modified Tyrode's buffer and platelets added (red). Chemokine (blue) is then applied at one end creating a concentration gradient and platelets visualised using an inverted light microscope (40x magnification).

Recordings were made using an Olympus Cell-R inverted light microscope (Finland), with a 40x magnification lens, running Xcellence software (Olympus) and regulated to 37°C in a heating chamber. Images were taken at 12 frames/minute for 8.33 minutes (i.e. 100 frames) and transferred to ImageJ (Fiji). 10-12 platelets were individually tracked over every frame using “Manual Tracking” plugin (Fabrice Cordelières, Institut Curie, Orsay, France), before transfer of tracks to Chemotaxis and Migration Tool (Ibidi, Germany). Data sets were calibrated to 5 seconds between frames with an x/y calibration of 6.2118 pixels/ μm as determined from Xcellence software and the microscope specifications. Data were graphically presented using spider plots, showing all tracks from a centre start point.

Various measurements of platelet chemotaxis were generated, which showcase different elements of cell migration. An average of the differences between the initial and final (x, y) values of migrating platelets, was termed the average centre of mass. The Euclidean distance was calculated as the straight line distance between the initial and final value of each platelet. The mean Euclidean distance (MED) was an average of all straight line distances of migrating platelets. The accumulated distance was a sum of the incremental distances moved by a platelet between each frame. The mean accumulated distance (MAD) was an average of the total distances migrated by platelets. The mean velocity was calculated as the MAD divided by time ($\mu\text{m}/\text{sec}$), of each platelet and averaged. The directionality represents the tendency of cells to migrate in a straight line and is calculated by dividing the MED by MAD. A directionality of 1 would indicate a straight line migration and a directionality of 0 would indicate random movement.

2.4.4 Human washed platelet preparation for in-vitro chemotaxis assay

Human peripheral venous blood was collected from healthy volunteers into ACD (1:9), in accordance with the Human Tissue Act 2004 and with local ethical approval from King's College London. The blood was centrifuged at 120g for 20 minutes. PRP supernatant was collected and the blood centrifuged again at 120g for 20 minutes, in order to collect the remaining PRP. PGE₁ (60 nM) was added to PRP before a centrifugation at 2200g for 10 minutes at 25°C, the supernatant was then aspirated off and the platelet pellet re-suspended in 1 ml RPMI 1640. PGE₁ (60 nM) was then added and washed platelets made up to 10 ml in RPMI 1640. This was followed by another centrifugation at 2200g for 10 minutes at 25°C, the supernatant was aspirated off and the platelet pellet re-suspended in 1 ml RPMI 1640. A small sample of washed platelets was added 1:100 to Stromatol and platelet concentration quantified using a haemocytometer and a phase contrast light microscope with 40x objective. Human washed platelets were diluted to 1×10^8 platelets/ml.

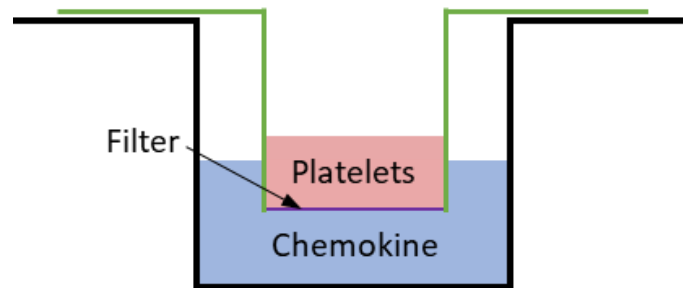


Figure 2.9: Transwell chemotaxis assay arrangement.

2.4.5 Characterisation experiments of transwell chemotaxis assay to measure human platelet motility to chemokines

24-well cell culture plates (Corning) were blocked with 1% BSA in RPMI 1640 for 1 hour. 600 µl of media or media with chemokine was added to the bottom well. Human platelets were prepared as previously described (section 2.4.4) and treated with CaCl₂ (2 mM) and ADP (100 nM), 10 minutes prior to being added to the top well (inserts). Cell culture inserts

(Falcon) with 3 μm pore sizes were positioned in the wells and 250 μl platelets subsequently added into the inserts (Figure 2.9). The plate was then incubated at 37°C for varying durations (time dependency was investigated as a variable), after which media from bottom chambers was collected 1:2 in Stromatol and quantified on a haemocytometer using a phase contrast light microscope (Zeiss Axiovert) with 40x objective lens.

2.5 Chemokine receptor expression

2.5.1 Chemokine receptor expression by flow cytometry

Mouse washed platelets from sham-sensitized and OVA-sensitized mice (section 2.3.2 and 2.4.1) were diluted to 1×10^7 platelets/ml in modified Tyrode's buffer. Saturating concentrations of anti-mouse CD41 fluorescein isothiocyanate (FITC) conjugated antibody and the relevant anti-mouse CCR1 PE, anti-CCR3 PE, anti-CCR4 PE or anti-CXCR4 PE conjugated antibodies were added to separate 10 μ l platelet samples. The relevant isotype controls (IgG2b κ FITC and IgG1 κ PE conjugated antibodies) were also prepared. After 30 minutes incubation at room temperature in a dark room, 500 μ l of PBS was added to each sample before running on a Beckman Coulter FC500 flow cytometer with CXP acquisition software (Beckman Coulter, UK). Gating was set on isotype control antibody samples, excluding $\leq 2\%$ of events.

2.5.2 Total protein assay

PRP from sham-sensitized and HDM-sensitized mice was separated from whole blood as described in section 2.4.1. PGE₁ (60 nM) was added to PRP and then centrifuged at 800g for 6 minutes with PPP aspirated off. The platelet pellet was resuspended in cell lysis buffer containing Protease inhibitor, Phosphatase inhibitor cocktail 2 and Phosphatase inhibitor cocktail 3 (Sigma Aldrich, UK). The bicinchoninic acid (BCA) protein assay kit was used to quantify levels of protein according to the manufacturer's instructions. A plate reader was used to measure absorbance (562 nm) and the levels of protein determined against BCA standards, with background values subtracted.

2.5.3 Measurements of chemokine receptor expression by western blot analysis

Lysed platelets were prepared as described in section 2.5.2. LDS sample buffer and sample reducing agent were added to 30 μ g of protein and made up to 10 μ l in sterile water. MOPS running buffer was used to fill the inner and outer chambers of the western blot tank, with antioxidant added to the middle chamber. Samples were denatured by heating for 10

minutes at 95°C and subsequently loaded into the gels, along with standards. The proteins were separated by gel electrophoresis at 200V, 3.00A and 300W for 50 minutes. The gel was then removed from the chamber and sandwiched between filter paper and transfer membrane. The tank was filled with transfer buffer containing 10% methanol and antioxidant. Along with additional filters and sponges, the membrane sandwich was placed into the tank and transferred at 30V, 3.00A, 300W for 70 minutes.

The membrane was blocked with 3% BSA in TBST for 1 hour, washed in TBST and incubated overnight with 2 µg/ml of anti-mouse CCR1, CCR3, CCR4 or CXCR4 polyclonal antibodies. The membrane was then washed in TBST and incubated with anti-rabbit IgG, HRP-linked antibody. 1 hour later the membrane was washed in TBST and incubated with ECL plus solution for 5 minutes and transferred into a photographic cassette. Chemiluminescence film was placed over the membrane and left to develop for between 5-15 minutes. Finally, the film was processed in an automated western blot processor.

When re-probing the membrane for a different protein, the membrane was incubated in stripping buffer (ThermoFisher Scientific) for 15 minutes, washed in TBST, blocked with 3% BSA in TBST and finally incubated with the next primary antibody overnight.

2.6 Immunohistochemistry and histology

2.6.1 *Mouse lung and cremaster muscle processing and sectioning*

Fixed lung and cremaster muscle tissues (section 2.3.6 and 2.3.9) were dehydrated and paraffin wax embedded. 5 µm sections were cut using a Leica RM2125 RT rotary microtome and mounted on histological Superfrost Plus slides.

2.6.2 *Mouse lung and cremaster muscle CD42b platelet stain, and polymorphonuclear cell staining*

Mouse lung and cremaster muscle sections were passed through xylene, rehydrated and immersed in 3% H₂O₂ in ethanol for 10 minutes. Sections were washed with tap water and immersed in sodium citrate buffer at 100°C for 10 minutes using a pressure cooker. Sections were then blocked with 1% BSA in PBS for 1 hour, washed with PBS and incubated with rabbit anti-human CD42b antibody diluted 1:500 in 1% BSA in PBS. Control sections were incubated with 1% BSA in PBS. After 1 hour, sections were washed with PBS and then incubated with anti-rabbit IgG (biotinylated) diluted 1:200 in 1% BSA in PBS. After 1 hour, sections were washed with PBS and incubated with Vectastain ABC HRP kit (components A and B diluted 1:200) for another hour. Sections were then washed with PBS and developed using DAB Peroxidase (HRP) Substrate Kit containing 0.3% H₂O₂ for 10 minutes, followed by a tap water wash. Sections were counterstained with haematoxylin for 30 seconds, differentiated through immersion in acid alcohol solution for 5 seconds and washed in tap water. Finally, sections were dehydrated and cleared in xylenes, before coverslipping with DPX mountant.

Due to haematoxylin counter staining, polymorphonuclear neutrophils (PMN) were distinguishable from other leukocytes and numerated in mouse lung sections (section 2.6.4).

2.6.3 *Mouse lung and cremaster muscle Luna stain for eosinophils*

Lung and cremaster muscle samples were processed as previously described (section 2.6.1) and the tissue sections passed through xylene and rehydrated. Sections were immersed

in Weigart's iron haematoxylin kit solution for 2.5 minutes, washed with distilled water and differentiated in acid alcohol solution for 8 seconds (Luna, 1968). Sections were washed with tap water and immersed in 0.5% Li_2CO_3 solution for 5 seconds. Following a tap water wash, sections were dehydrated and cleared through xylenes before cover slipping with DPX mountant.

2.6.4 *Quantification of platelets, eosinophils and PMNs in mouse lung and cremaster muscle sections*

Images of sections stained for platelet CD42b antigen and Luna eosinophil stained sections, were captured using a 40x objective lens on a Leica DM 2000 LED bright field microscope with Leica DFC295 camera. Platelets or eosinophils in extravascular compartments of cremaster muscle sections were numerated and expressed per mm^2 of cremaster muscle tissue using ImageJ analysis software. In lung tissue samples, the number of extravascular platelets, eosinophils or PMNs in the airway walls were quantified and expressed per mm length of airway walls.

2.6.5 *Human lung CD42b platelet stain*

Post mortem human lung tissue was a generous gift from Professor Jan Shute (University of Portsmouth, UK). Formalin-fixed paraffin-embedded slides of human lung samples from control, non-fatal and fatal asthma patients were processed and sectioned at the Airway Disease Biobank (Department of Pulmonary and Sleep Medicine, West Australian Sleep Disorder Research Institute, Sir Charles Gardiner Hospital, Nedlands, Australia). Staining for the platelet CD42b antigen was performed using the same protocol as in section 2.6.2. However, anti-human CD42b antibody was diluted 1:200 and the DAB developing step replaced. Sections were instead developed in NovaRED Peroxidase (HRP) Substrate (Vector, UK) solution for 20 minutes, washed in tap water and the remainder of the protocol followed.

2.7 Statistical analysis

A Student's t-test was used when comparing between single independent measurements from two groups. When comparing between three or more groups a one-way analysis of variance (ANOVA) or a repeated measures one-way ANOVA test was used, with Dunnett's multiple comparison post hoc test. A two-way repeated measures ANOVA was used when comparing the effects of two independent variables on three or more groups. All data are presented as Mean \pm Standard Error of the mean (SEM). A $P < 0.05$ between individual groups was considered significant. Data from the microplate chemotaxis assay was presented as a chemotaxis index. This was calculated by dividing the number of platelets migrated by its respective control. Statistical analyses were performed on Prism 6.0 (GraphPad Software, USA).

**Chapter 3 Results I: Presence of
platelets in human lung samples and
expression of platelet chemokine
receptors in sham vs allergen-
sensitized mice**

3.1 Immunohistochemical staining of platelets in human lung sections

Platelets have been demonstrated in extravascular compartments of bronchial biopsy samples and in bronchoalveolar lavage fluid from patients with asthma (Metzger et al., 1987; Jeffery et al., 1989). However, these early studies did not quantify platelet recruitment, nor did they identify their precise anatomical localisation. Platelets have also been demonstrated to migrate into lungs of allergen-sensitized mice after allergen exposure (Pitchford et al., 2008). The consequences of platelet migration following an asthma attack are unknown, but using sections of lung biopsy samples from patients with asthma (but who died of other causes), and patients who died following an asthma attack, antigen specific immunohistochemistry allows platelet quantification and location to be determined.

Sections of lung biopsies from healthy, non-fatal asthmatic and fatal asthmatic patients were immunohistochemically stained using a platelet specific anti-human CD42b antibody. Single non-aggregated platelets were observed in the alveolar walls and in the alveolar space of lung sections taken from patients who died from asthma (Figure 3.1A). The number of platelets in the lungs of non-fatal asthmatic patients compared to healthy patients was slightly elevated, although not significantly (healthy vs. non-fatal asthmatic: 36.3 ± 6.4 platelets/mm² vs. 48.8 ± 7.9 platelets/mm²) (Figure 3.1B). There was an elevated number of platelets in fatal asthmatic patients compared to healthy patients (healthy vs. fatal asthmatic: 36.3 ± 6.4 platelets/mm² vs. 88.6 ± 12.2 platelets/mm², $P < 0.01$). Interestingly, there were increased CD42b positive platelet staining events in fatal asthmatic compared with non-fatal asthmatic patients (non-fatal asthmatic vs. fatal asthmatic: 48.8 ± 7.9 platelets/mm² vs. 88.6 ± 12.2 platelets/mm², $P < 0.05$), suggesting airway provocation causes platelet recruitment to the lungs through non-aggregatory activation pathways.

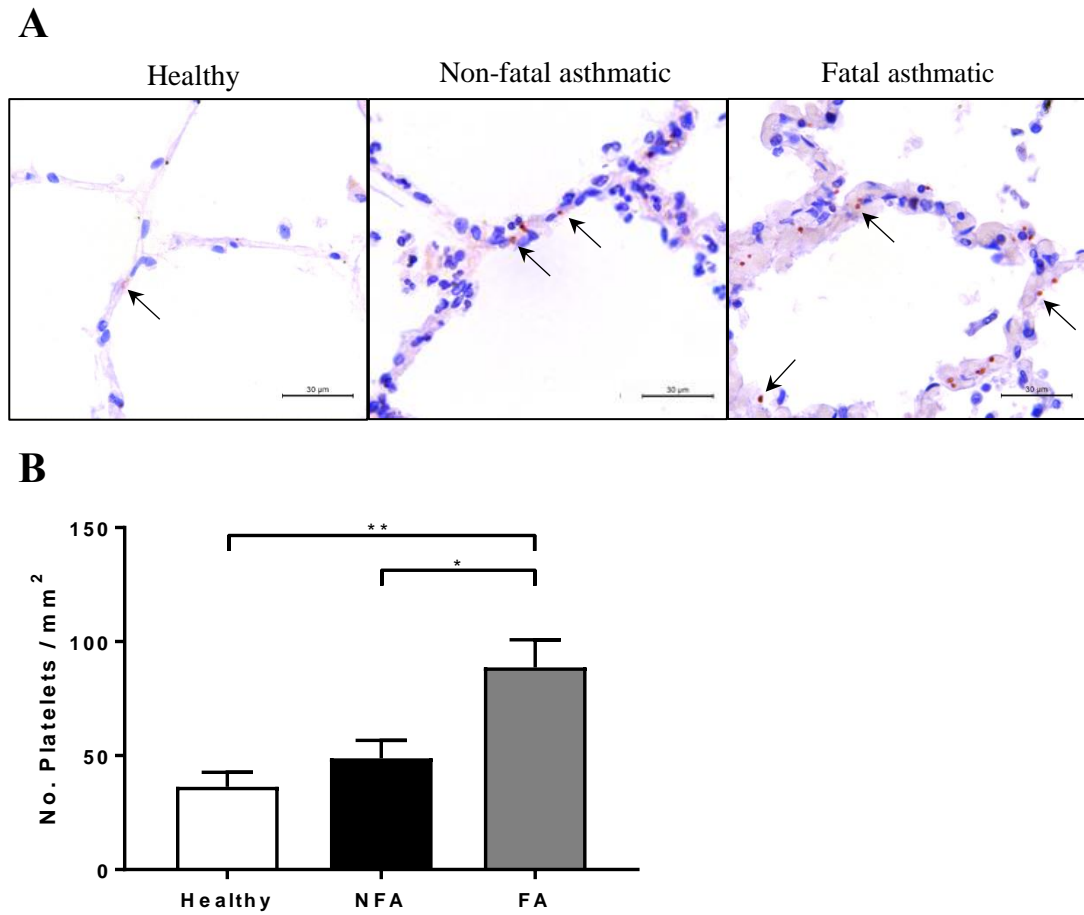


Figure 3.1: Immunostaining CD42b+ platelets in human lung samples.

Formalin-fixed, paraffin-embedded slides of post mortem human lungs from healthy, non-fatal asthmatic (NFA) and fatal asthmatic (FA) patients were immunostained with platelet specific anti-human CD42b antibody. A- representative images of healthy, non-fatal asthmatic and fatal asthmatic patients magenta CD42b+ platelet staining events (highlighted with arrows). B- the number of platelet staining events in healthy, non-fatal asthmatic (NFA) and fatal asthmatic (FA) lung sections per mm², from 10 fields of view per section. Data presented as mean ± SEM, n = 8-10, * = $P < 0.05$ and ** = $P < 0.01$.

Unfortunately, sections of lung containing airway wall occurred with insufficient frequency to further elucidate the localisation of platelets to other lung structures. However, platelets were observed around the airway walls in histological sections from patients with asthma, and patients who died of non-fatal asthma (Figure 3.2). Single platelets and platelets associated with leukocytes, were also observed within the airways in non-fatal asthmatic and fatal asthmatic patients (Figure 3.2).

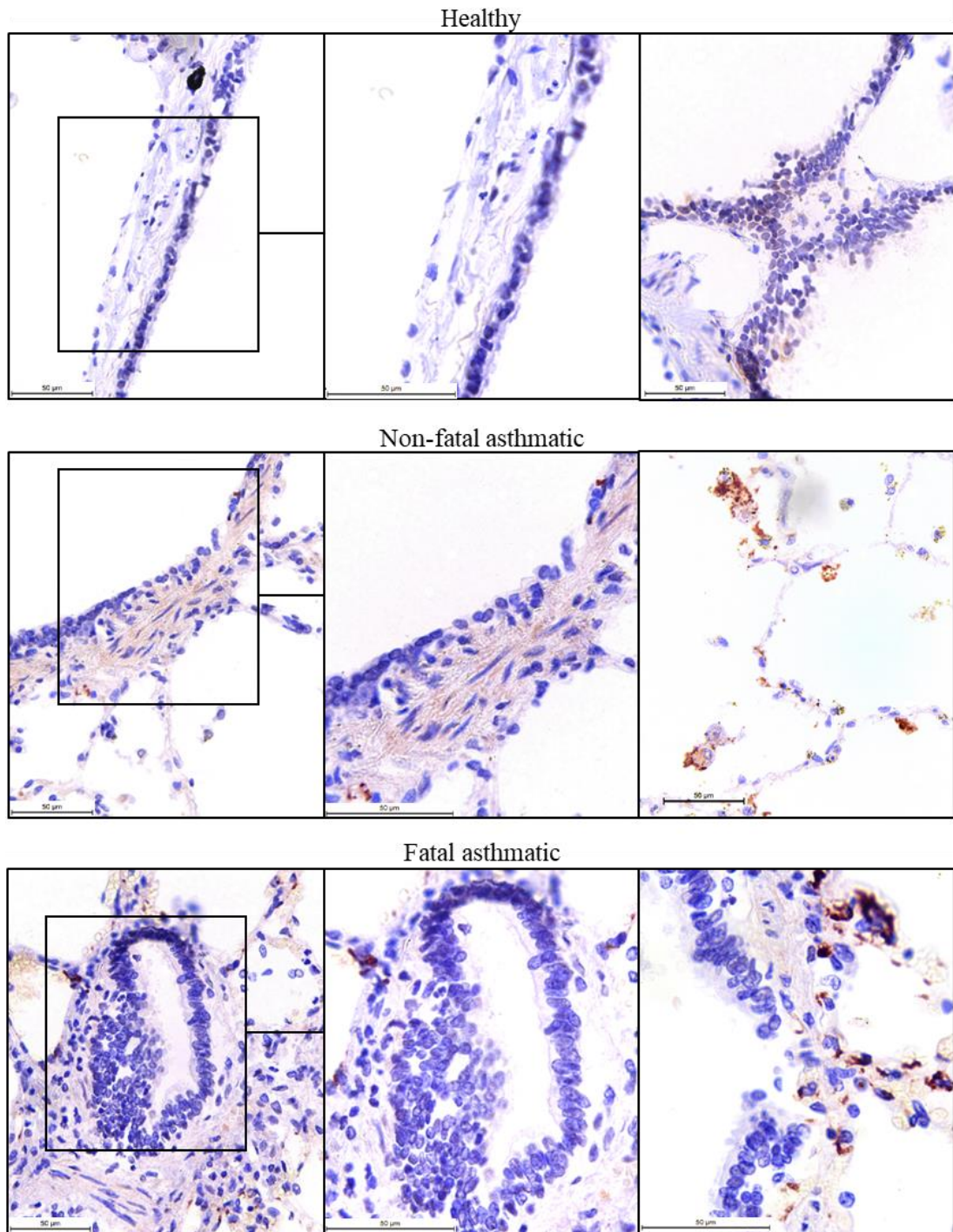


Figure 3.2: Representative immunostaining of CD42b+ platelets in airway walls.

Formalin-fixed, paraffin-embedded slides of post mortem human lungs from healthy, non-fatal asthmatic and fatal asthmatic patients were immunostained with platelet specific anti-human CD42b antibody. Presented are representative images of airway walls from healthy, non-fatal asthmatic and fatal asthmatic patients with magenta CD42b+ platelet staining events.

3.2 Platelet chemokine receptor expression in sham and allergen-sensitized mice

3.2.1 Platelet chemokine receptor expression in OVA-sensitized mice by flow cytometry

The mechanisms that underlie platelet migration to sites of allergic inflammation are unknown. Whilst pulmonary platelet recruitment, and migration towards an experimental allergen (OVA) has been reported to be platelet FcεR1-dependent, it is not understood how chemokines might be involved in platelet tissue localization in the context of allergic inflammation (Pitchford et al., 2008). Human platelets express CC chemokine receptors CCR1, CCR3 and CCR4, and CXC chemokine receptor CXCR4 (Kowalska et al., 1999; Clemetson et al., 2000; Gear et al., 2001), which may function in a similar manner to chemokine receptors expressed on leukocytes that lead to leucocyte recruitment and migration (Zhang et al., 2009). Human platelet chemotaxis was observed *in-vitro* through CXCR4 receptor activation via stromal cell-derived factor 1α (SDF-1α), demonstrating platelet chemokine receptor functionality (Kraemer et al., 2010). To interrogate the roles of platelet chemokine receptors in allergic responses, platelets harvested from mice sensitized to experimental allergen were used to investigate whether the sensitization process might affect platelet chemokine receptor expression and chemotaxis (Kuehn and Gilfillan, 2007).

Washed platelets from sham-sensitized and OVA-sensitized mice (on days 0, 4, and 11) were harvested from peripheral blood on day 14. Flow cytometry was then conducted to investigate the effects of allergen-sensitization on chemokine receptor expression. Representative flow cytometric traces of platelet specific anti-mouse CD41-FITC conjugated antibody with anti-mouse IgG-PE conjugated antibody isotype control and anti-mouse CD41-FITC conjugated antibody with anti-mouse CCR1-PE conjugated antibody, show a separate

population of platelets that express CCR1 (Figure 3.3C), which was absent in the isotype control (Figure 3.3A).

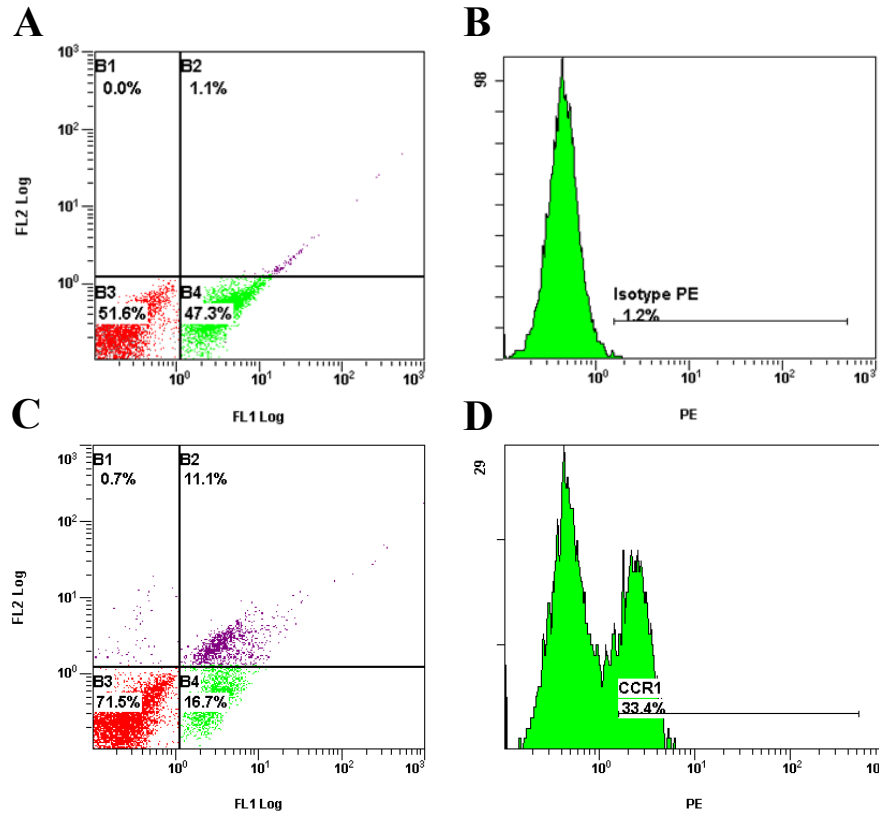


Figure 3.3: Representative flow cytometry traces of isotype IgG-PE and CCR1-PE.

Washed platelets taken on day 14 from sham-sensitized and OVA-sensitized mice (30 μ g OVA on days 0, 4 and 11) chemokine receptor expression was measured by flow cytometry. Presented are representative traces of OVA-sensitized mouse washed platelets, incubated with anti-mouse IgG-PE conjugated antibody and anti-mouse CD41-FITC conjugated antibody (A and B), and with anti-mouse CCR1-PE conjugated antibody and anti-mouse CD41-FITC conjugated antibody (C and D). A and C display density plots of FL1 (FITC) plotted against FL2 (PE). B and D display histograms of events recorded in PE gate.

Each of the four chemokine receptors CCR1, CCR3, CCR4 and CXCR4 that are expressed on human platelets, were also expressed on mouse platelets (Figure 3.3 and 3.4). CCR3 receptor expression trended towards an increase in OVA-sensitized mice compared to sham-sensitized mice, although this effect was not significant (sham vs. OVA: 10.6 ± 7.6 vs. 19.1 ± 9.5 %, Figure 3.5A). No similar elevated expression was noted in OVA-sensitized mice compared to sham-sensitized mice for CCR1, CCR4, or CXCR4 (Figure 3.5A). An indication

of the number of chemokine receptors expressed per individual platelet (or event), termed mean fluorescence intensity (MFI), was variable between animals making it difficult to determine differences between sham-sensitized and OVA-sensitized mice (Figure 3.5B).

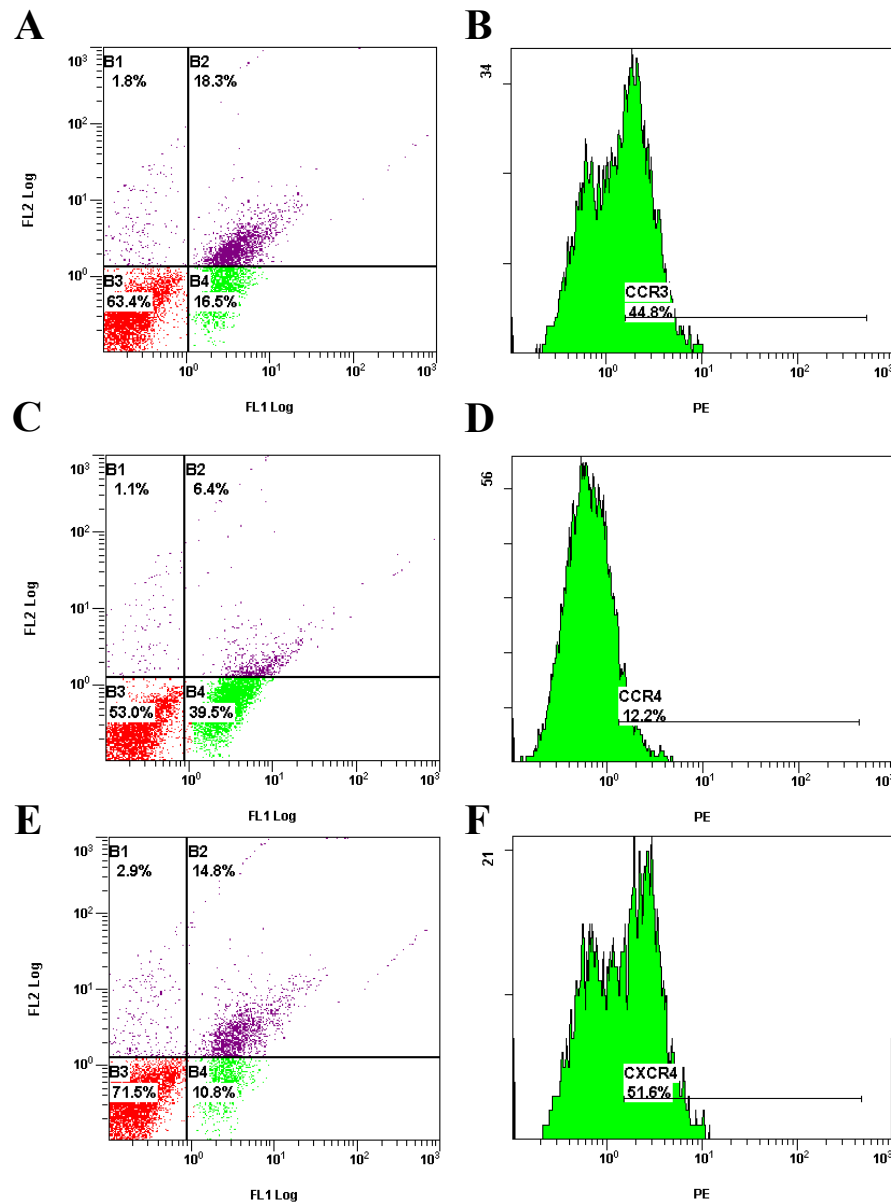


Figure 3.4: Representative flow cytometry traces of CCR3-PE, CCR4-PE and CXCR4-PE.

Washed platelets taken on day 14 from sham-sensitized and OVA-sensitized mice (30 μ g OVA on days 0, 4 and 11), chemokine receptor expression was measured by flow cytometry. Presented are representative samples containing anti-mouse CD41-FITC conjugated antibody with either anti-mouse CCR3-PE conjugated antibody (A and B), anti-mouse CCR4-PE conjugated antibody (C and D) or anti-mouse CXCR4-PE conjugated antibody (E and F). A, C and E display density plots of FL1 (FITC) plotted against FL2 (PE). B, D and F display histograms of events recorded in PE gate.

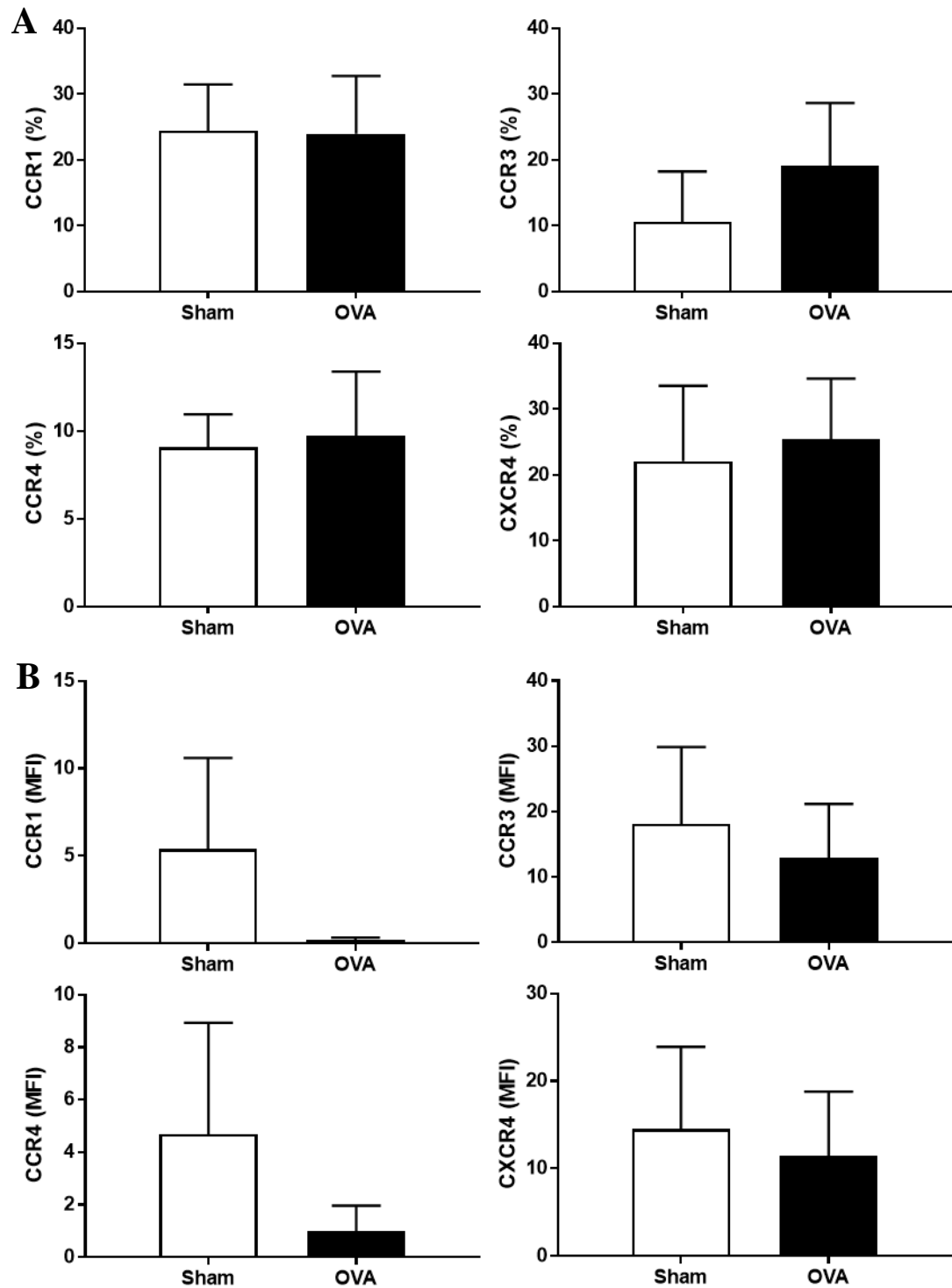


Figure 3.5: The effects of OVA-sensitization of mice on platelet chemokine receptor expression and MFI.

Washed platelets taken on day 14 from sham-sensitized and OVA-sensitized mice (30 μ g OVA on days 0, 4 and 11), were incubated for 30 minutes with anti-mouse CD41 FITC and either anti-mouse CCR1-PE conjugated antibody, anti-mouse CCR3-PE conjugated antibody, anti-mouse CCR4-PE conjugated antibody conjugated antibody or anti-mouse CXCR4-PE conjugated antibody. Chemokine receptor expression was measured by flow cytometry. A- percentage chemokine receptor expression on CD41+ platelet population. B- mean fluorescence intensity. Data presented as mean \pm SEM and n = 8.

3.2.2 Western blot analysis of CCR1, CCR3, CCR4 and CXCR4 platelet chemokine receptors in HDM-sensitized mice

The effects of allergen-sensitization on platelet surface expression of CCR1, CCR4 and CXCR4 receptors was minimal. However, it is possible that translational events of chemokine receptor proteins may increase after allergen-sensitization, leading to increased platelet chemokine receptor protein content, which remains internalised until platelet activation. Furthermore, flow cytometric data revealed a doubling in CCR3 receptor expression in mice sensitized to experimental allergen. Western blot analysis was therefore conducted on lysed

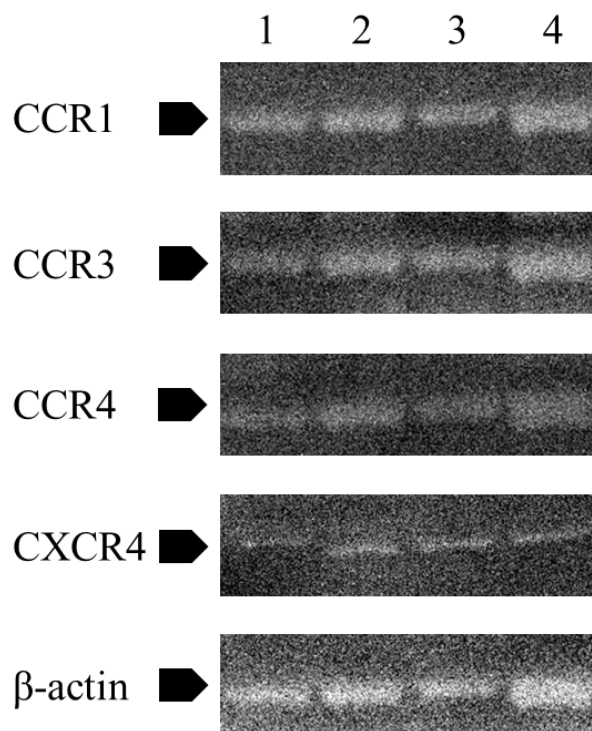


Figure 3.6: Representative immunoblots of CCR1, CCR3, CCR4 and CXCR4 receptor expression.

Platelet rich plasma taken on day 14 from sham-sensitized and HDM-sensitized mice (25 µg HDM on days 0, 1, 2, 3, 4, 7, 8, 9, 10, and 11) was lysed in cell lysis buffer, and protein concentration determined using the BCA assay. 30 µg of lysed platelet protein was loaded into gels, before gel electrophoresis and transfer steps. Membranes were incubated with anti-mouse CCR1, CCR3, CCR4, CXCR4 or β-actin antibodies. Membranes were then incubated with anti-rabbit IgG HRP-linked secondary antibody, followed by ECL plus solution, before chemiluminescence imaging. Representative immunoblots from sham-sensitized (lanes 1 and 2) and HDM-sensitized (lane 3 and 4) mice are presented.

platelets to investigate internal protein amounts, to corroborate results produced from flow cytometry. However, the sensitization model for mice was changed from the OVA to the HDM-sensitization model, due to the formation of $Al(OH)_3$ deposits in the cremaster muscle, which is further described in section 5.1.1.

Washed platelets from sham-sensitized and HDM-sensitized mice (on days 0, 1, 2, 3, 4, 7, 8, 9, 10 and 11) were harvested from peripheral blood on day 14. Platelet CCR1, CCR3, CCR4 and CXCR4 receptor expression was confirmed via western blot analysis (Figure 3.6).

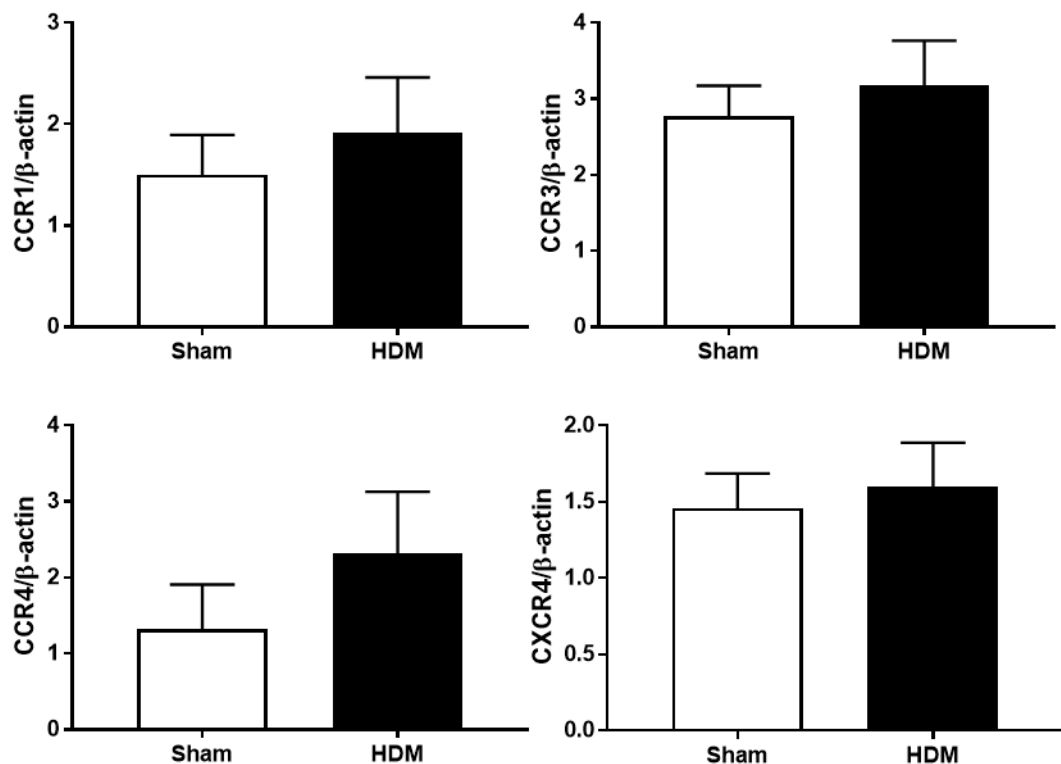


Figure 3.7: The effects of HDM-sensitization of mice on platelet chemokine receptor expression.

Platelet rich plasma taken on day 14 from sham-sensitized and HDM-sensitized mice (25 μ g HDM on days 0, 1, 2, 3, 4, 7, 8, 9, 10, and 11) was lysed in cell lysis buffer, and protein concentration determined using the BCA assay. 30 μ g of lysed platelet protein was loaded into gels, before gel electrophoresis and transfer steps. Membranes were incubated with anti-mouse CCR1, CCR3, CCR4, CXCR4 or β -actin antibodies. Membranes were then incubated with anti-rabbit IgG HRP-linked secondary antibody, followed by ECL plus solution, before chemiluminescence imaging and band analysis using ImageJ. Data are expressed per β -actin in each sample lane. Data presented as mean \pm SEM and n = 6-7.

Chemokine receptor expression was similar in both sham-sensitized and HDM-sensitized mice, with a tendency for increased levels in HDM-sensitized groups (Figure 3.7). Western blot analysis shows that HDM-sensitization of mice does not cause an increase in total platelet chemokine receptor protein production compared to sham-sensitized mice.

In summary, these results show CCR1, CCR3, CCR4 and CXCR4 receptor expression on mouse platelets that have previously been reported on human platelets and demonstrate that sensitization of mice to experimental allergen does not significantly alter chemokine receptor expression on the surface of platelets or platelet chemokine receptor protein production.

**Chapter 4 Results II: The effects of
allergen-sensitization on chemokine
receptor induced platelet chemotaxis
and *in-vitro* platelet chemotaxis assay
model development**

4.1 Microplate chemotaxis assay

Following platelet chemokine receptor expression presented in Chapter 3, the ability of these receptors to induce platelet chemotaxis was investigated *in-vitro*. Platelet migration has been observed using a modified Boyden chamber, where allergen-sensitization of mice caused increased platelet chemotaxis towards allergen via an FcεRI-dependent mechanism (Pitchford et al., 2008). FcεRI and chemokine receptors on mast cells have synergistic roles in mast cell degranulation and chemotaxis, suggesting a possible mechanism through which allergen-sensitization may impact platelet chemotactic responses (Kuehn and Gilfillan, 2007). Whilst platelets have been reported to undergo chemotaxis towards other chemoattractants and chemokines, notably fMLP and SDF-1α, the influence of allergen-sensitization on platelet chemotaxis towards chemokines has not been reported (Czapiga et al., 2005; Kraemer et al., 2010). The ability of MIP-1α, eotaxin, MDC, and SDF-1α, which bind CCR1, CCR3, CCR4 and CXCR4 chemokine receptors respectively (D'Apuzzo et al., 1997; Imai et al., 1997; Pease et al., 1998), to cause human and mouse platelet chemotaxis, and the influence of allergen-sensitization was therefore investigated.

4.1.1 An investigation into the effects of allergen-sensitization on platelet chemotaxis

The Neuro Probe ChemoTx microplate (microplate chemotaxis assay, 2 μm pore size) was initially used to study the effects of allergen-sensitization on platelet chemotaxis. On days 0, 4 and 11, sham mice received 0.4 ml PBS with 0.1 M Al₂(OH)₃ i.p. and OVA-sensitized mice received 30 μg OVA in 0.4 ml PBS with 0.1 M Al₂(OH)₃. On day 14, washed platelets prepared from sham and OVA-sensitized mice were stimulated with ADP (0.1 μM), 10 minutes before microplate chemotaxis assay experiments, because previous data from our laboratory reported that platelet priming via the P2Y₁ receptor provides a robust response towards fMLP and MDC (Amison et al 2015; 2018).

MIP-1α, a ligand of the CCR1 receptor, did not induce platelet chemotaxis at either 10 nM or 100 nM (Figure 4.1A). Eotaxin, a ligand of the CCR3 receptor caused a concentration

dependant increase on chemotaxis in platelets harvested from OVA-sensitized mice ($P < 0.01$), with a significant difference between sham and OVA-sensitized groups at 100 nM (chemotactic index: sham vs. OVA: 1.2 ± 0.1 vs. 1.6 ± 0.2 , $P < 0.05$) (Figure 4.1B). MDC, a ligand of the CCR4 receptor caused a significant concentration dependent effect on platelet chemotaxis ($P < 0.05$), with no differences between sham and OVA-sensitized groups (Figure 4.1C). SDF-1 α , a ligand of the CXCR4 receptor, appeared to have no effect on platelet chemotaxis (Figure 4.1D).

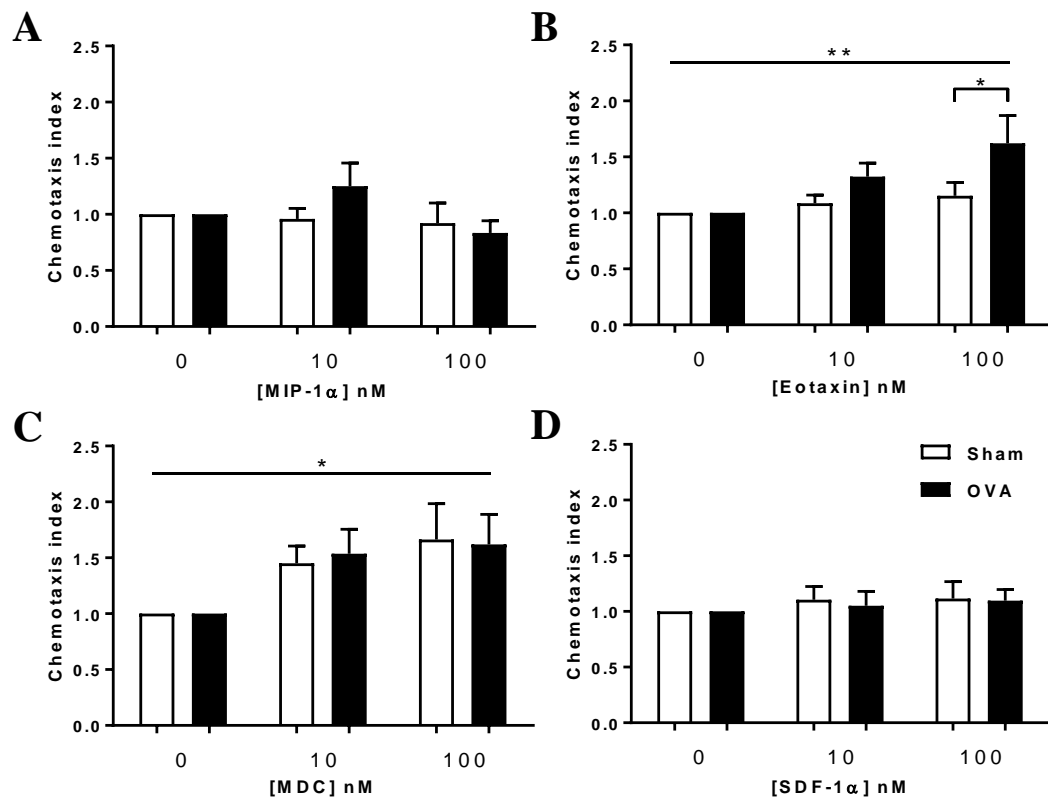


Figure 4.1: The effects of MIP-1 α , eotaxin, MDC, and SDF-1 α on platelet chemotaxis in OVA-sensitized mice.

Washed platelets were taken on day 14 from sham-sensitized and OVA-sensitized mice (30 μ g OVA on days 0, 4 and 11). A range of MIP-1 α , eotaxin, MDC, and SDF-1 α concentrations were added to the bottom wells, before washed platelets were treated with ADP (0.1 μ M) and added to the top well of a chemotaxis chamber. The chamber was then incubated for 120 minutes, before the quantification of platelets in the bottom well. Responses of sham-sensitized and OVA-sensitized mice to MIP-1 α (A), eotaxin (B), MDC (C), and SDF-1 α (D) are displayed. Data was analysed as a chemotactic index and expressed as mean \pm SEM, $n = 8$, * = $P < 0.05$ and ** = $P < 0.01$.

4.1.2 An investigation into the effects of allergen challenge on platelet chemotaxis

OVA-sensitized challenged mice have exhibited platelet migration to lung tissue (Pitchford et al., 2008). Thus, it was hypothesised that allergen challenge may enhance the platelet chemotactic responses observed in section 4.1.1. Sham and OVA-sensitized mice were challenged with 30 µg aerosolised OVA on days 14, 15 and 16, and blood collected on day 17. MIP-1α was again ineffective at causing platelet chemotaxis (Figure 4.2A), suggesting that the platelet CCR1 receptor is not involved in platelet migration in the conditions measured here, therefore the CCR1 receptor was not investigated further within this thesis.

The effect of eotaxin on platelet chemotaxis followed a similar trend to effects observed in OVA-sensitized mice. Platelet migration at 10 nM and 100 nM eotaxin trended toward an increase in OVA-sensitized and challenged groups, although this effect was not significant, and therefore there was also no difference in platelet chemotaxis towards eotaxin between sham and OVA-sensitized mice (Figure 4.2B). MDC produced a significant concentration effect on platelet chemotaxis irrespective of sensitization status ($P < 0.05$) (Figure 4.2C). SDF-1α trended toward an increase in platelet chemotaxis that appeared elevated in OVA-sensitized and challenged groups, however this effect was not significant (Figure 4.2D). This suggests a potential role of the CXCR4 receptor after allergen challenge that was not observed in OVA-sensitized mice without subsequent allergen exposure. The results of eotaxin and MDC were decreased to those seen in OVA-sensitized mice without subsequent allergen exposure, suggesting OVA challenge does not further elevate *in-vitro* platelet chemotaxis, rather this could be interpreted that CCR3 and CCR4 responsive platelets had been recruited to the lungs *in-vivo*, leaving a ‘non’ responsive circulating platelet population.

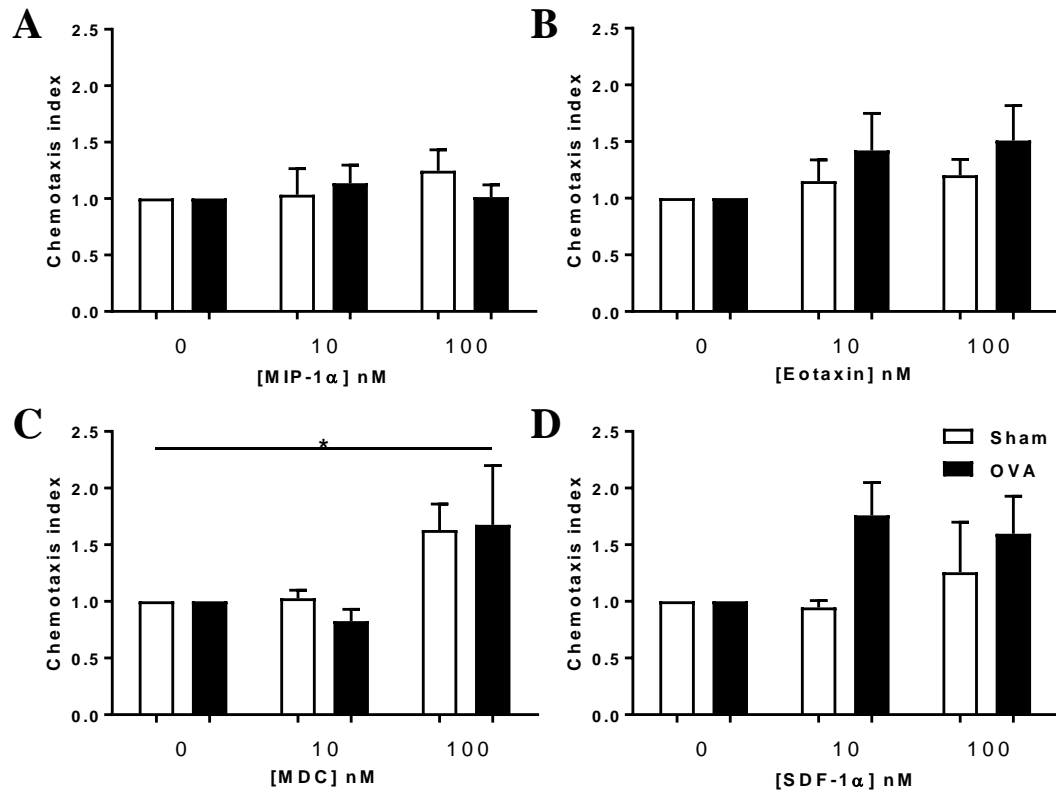


Figure 4.2: The effects of MIP-1 α , eotaxin, MDC, and SDF-1 α on platelet chemotaxis in OVA-sensitized and OVA-challenged mice.

Washed platelets were taken on day 17 from sham-sensitized and OVA-sensitized mice (30 μ g OVA on days 0, 4 and 11), challenged with aerosolised OVA (30 mg/ml OVA on days 14, 15 and 16). A range of MIP-1 α , eotaxin, MDC, and SDF-1 α concentrations were added to the bottom wells, before washed platelets were treated with ADP (0.1 μ M) and added to the top well of a chemotaxis chamber. The chamber was then incubated for 120 minutes, before the quantification of platelets in the bottom well. Responses of sham-sensitized and OVA-sensitized mice to MIP-1 α (A), eotaxin (B), MDC (C), and SDF-1 α (D) are displayed. Data was analysed as a chemotaxis index and expressed as mean \pm SEM, $n = 8$ and $* = P < 0.05$.

Thus, using the microplate chemotaxis assay, mouse platelet chemotaxis was demonstrated to eotaxin and MDC via CCR3 and CCR4 receptor stimulation, respectively. Interestingly, platelet chemotaxis towards eotaxin, was elevated in allergen-sensitized groups, suggesting that CCR3 might be involved in platelet recruitment and motility during allergic inflammatory responses. It is also noted that whilst platelet chemotaxis could be demonstrated using the Neuro Probe ChemoTx microplates, the chemotactic indices were low, especially compared to the motility of leukocytes. Further experiments were therefore conducted to investigate if different methodologies could improve the assay.

4.2 Transwell chemotaxis assay

4.2.1 Platelet chemotaxis towards fMLP using a transwell assay design

In-vitro human platelet chemotaxis has been shown towards fMLP and SDF-1 α , using the microplate chemotaxis assay, and fibrinogen coated chamber slides, respectively (Czapiga et al., 2005; Kraemer et al., 2010). The transwell chemotaxis assay features Falcon cell culture inserts in a 24-well plate, to create a chemotactic gradient. Such a system provides more flexibility with assay development as volumes in top and bottom wells can be adjusted. Previous studies in house, demonstrated that increasing the lower well volume from 450 μ l to 600 μ l, increases the precision of the assay. The transwell chemotaxis assay was therefore adopted to try to improve assay conditions for investigating the effects of chemokine receptor stimulation, initially on human platelet chemotaxis. The assay would then be used to investigate further the role of allergen-sensitization on platelet chemotaxis using platelets from allergen-sensitized mice, after investigating the effects of eotaxin, MDC, and SDF-1 α on human platelet chemotaxis.

A concentration response to fMLP in human washed platelets demonstrated a significant difference between control and 30 nM fMLP (control vs 30 nM fMLP: $6.3 \pm 0.1 \times 10^5$ platelets/ml vs $9.9 \pm 1.4 \times 10^5$ platelets/ml, $P < 0.05$), indicating platelets are sensitive to fMLP in this assay (Figure 4.3A). The results were also expressed as a chemotactic index (Figure 4.3B). Stimulating platelets may lead to increased activity and random movement. This phenomenon may manifest in increasing the number of platelets that move into the chemokine well. In order to eliminate the chance that platelet migration is due to increased chemokinetic activity and not chemotaxis, a chemokinetic control group (CKC) with equal concentrations of fMLP in the top and bottom wells was used in transwell chemotaxis assay studies. Thus, platelet chemotaxis towards fMLP was ablated in these conditions (Figure 4.3A & 4.3B), suggesting that platelet chemotaxis towards fMLP in the bottom well alone was the result of directed rather than random movement. In order to use fMLP as a positive control

when testing other stimuli, a greater window of effect between negative and positive control is required.

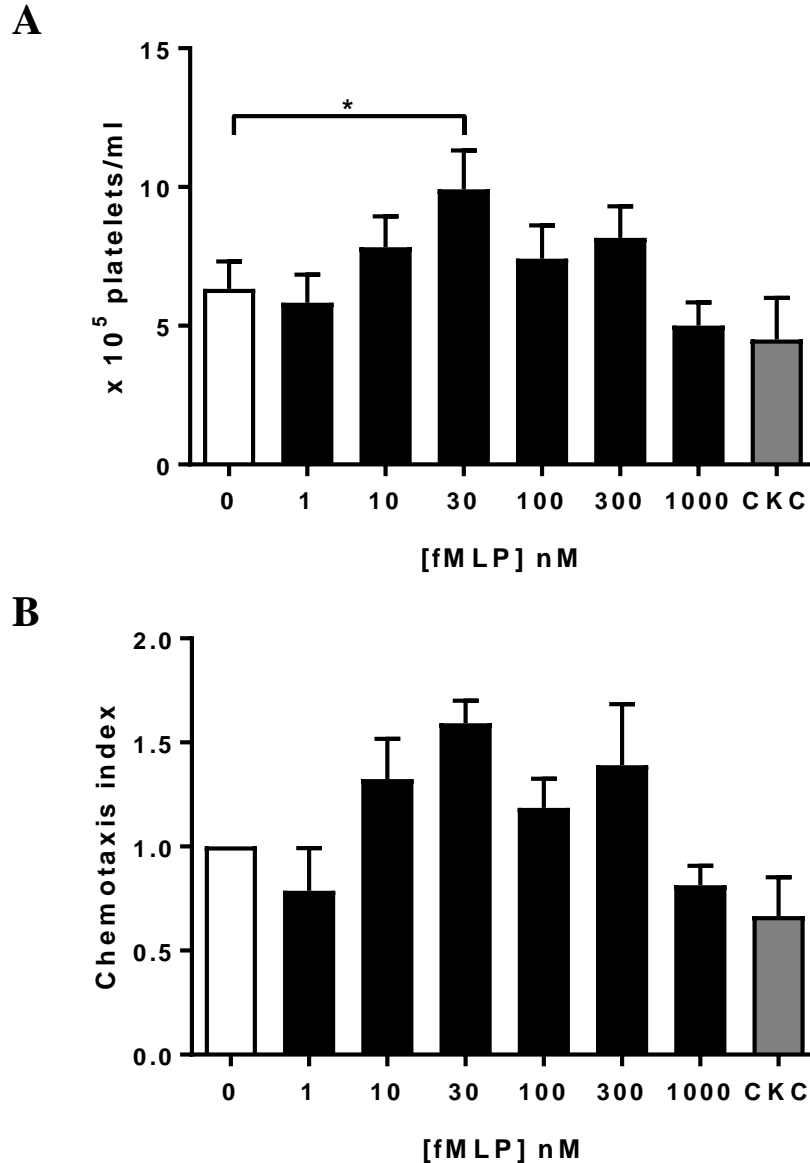


Figure 4.3: Human platelet chemotaxis fMLP concentration response.

Washed platelets were prepared from citrated human blood. A range of fMLP concentrations were added to the bottom chamber, before the transwell insert was placed into the bottom chamber, and platelets treated with CaCl_2 (2 mM) and ADP (0.1 μM), subsequently added to the insert. The whole chamber incubated for 90 minutes. A chemokinetic control group (CKC) had equimolar concentrations of fMLP (100 nM) in top and bottom wells. Following incubation, the number of platelets in the bottom well was enumerated. Data are plotted as the number of platelets/ml (A) and as a chemotaxis index (B). Data are expressed as mean \pm SEM, $n = 6$ and $* = P < 0.05$.

4.2.2 *The effects of incubation times on platelet migration in the transwell chemotaxis assay*

In order to establish a more defined response to fMLP, the effects of different incubation times on platelet chemotaxis was investigated. Based on the previous incubation time of 90 minutes, earlier and later incubation time points of 45 minutes and 180 minutes were used. The CKC was adjusted from 100 nM to 30 nM fMLP in top and bottom chambers, because 30 nM fMLP produced the greatest response in section 4.2.1.

A 45 minute incubation period caused significant differences in platelet chemotaxis between 0 nM and 30 nM fMLP (0 nM vs 30 nM fMLP: $5.18 \pm 0.48 \times 10^5$ platelets/ml vs $13.57 \pm 2.13 \times 10^5$ platelets/ml, $P < 0.01$), with a 2.4 fold increase in platelet chemotaxis (Figure 4.4A). Similarly, a 90 minute incubation period caused platelet chemotaxis between 0 nM and 30 nM fMLP to significantly increase (0 nM vs 30 nM fMLP: $9.57 \pm 2.33 \times 10^5$ platelets/ml vs $20.14 \pm 6.3 \times 10^5$ platelets/ml, $P < 0.05$), with a 2.1 fold increase in platelet chemotaxis (Figure 4.4B). Finally, a 180 minute incubation period caused a 1.7 fold increase in platelet chemotaxis between 0 nM and 30 nM fMLP (0 nM vs 30 nM fMLP: $20.29 \pm 6.05 \times 10^5$ platelets/ml vs $34 \pm 7.2 \times 10^5$ platelets/ml, $P < 0.001$) (Figure 4.4C). The largest platelet chemotaxis window between 0 nM and 30 nM fMLP was produced after a 45 minute incubation period, which was subsequently used in later studies.

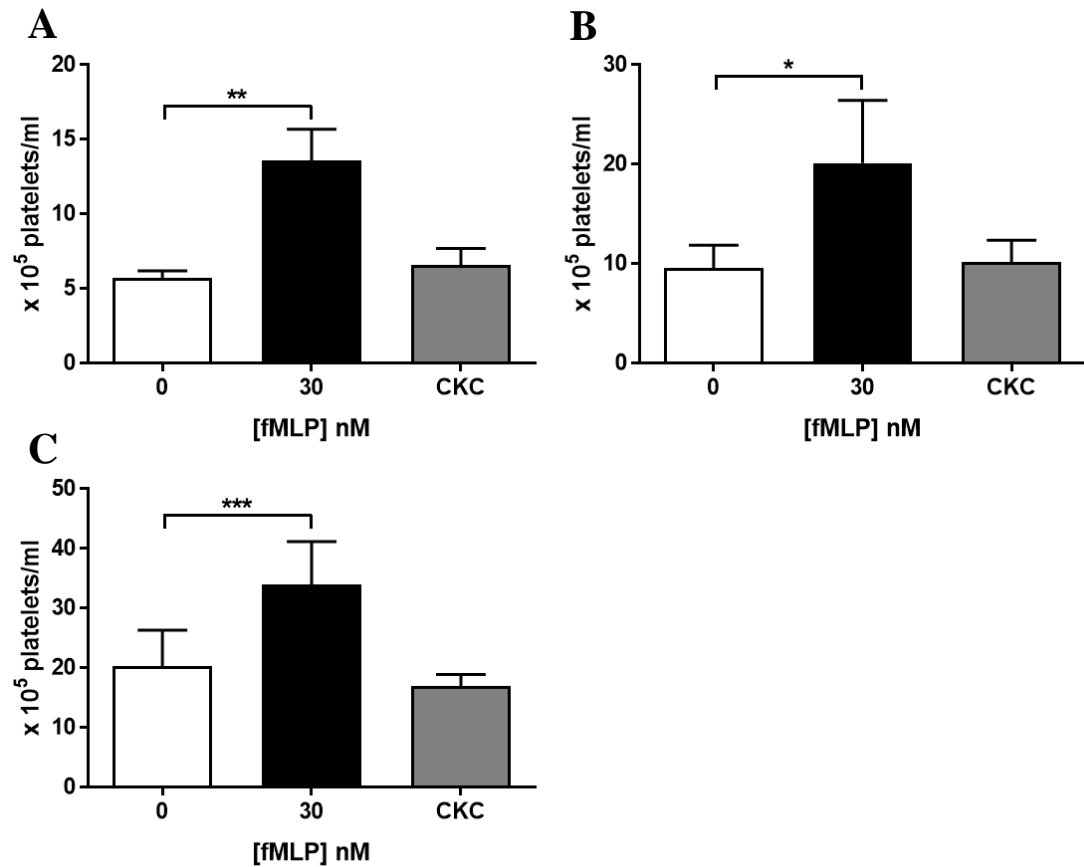


Figure 4.4: The effects of different incubation times on platelet chemotaxis to fMLP.

Washed platelets were prepared from citrated human blood. 0 nM or 30 nM fMLP was added to the bottom chamber, before the transwell insert was placed into the bottom chamber, and platelets treated with CaCl_2 (2 mM) and ADP (0.1 μM), subsequently added to the insert. A chemokinetic control group (CKC) had equimolar concentrations of fMLP (30 nM) in top and bottom wells. The chambers were incubated for 45 minutes (A), 90 minutes (B) or 180 minutes (C). Following incubation, the number of platelets in the bottom well was enumerated. Data was plotted as the number of platelets/ml. Data expressed as mean \pm SEM, $n = 6$ and $* = P < 0.05$.

4.2.3 fMLP concentration response with optimized incubation time

In order to investigate the optimum concentration of fMLP to act as a positive control in future experiments, a concentration response to fMLP was repeated using a 45 minute incubation period. Significant differences were observed in platelet chemotaxis when comparing 0 nM to 30 nM fMLP (0 nM vs 30 nM fMLP: $1.6 \pm 0.2 \times 10^5$ platelets/ml vs $4.0 \pm 0.5 \times 10^5$ platelets/ml, $P < 0.001$) and to 100 nM fMLP (0 nM vs 100 nM fMLP: $1.6 \pm 0.2 \times 10^5$ platelets/ml vs $2.9 \pm 0.6 \times 10^5$ platelets/ml, $P < 0.01$) (Figure 4.5A). When plotted as a chemotaxis index, there was a significant difference between 0 nM and 30 nM fMLP

(chemotaxis index: control vs. 30 nM fMLP: 1.0 ± 0.0 vs. 3.0 ± 0.7 , $P < 0.001$) (Figure 4.5B).

30 nM fMLP was subsequently used as a positive control in transwell chemotaxis assay investigations, as it produced the greatest platelet chemotactic response.

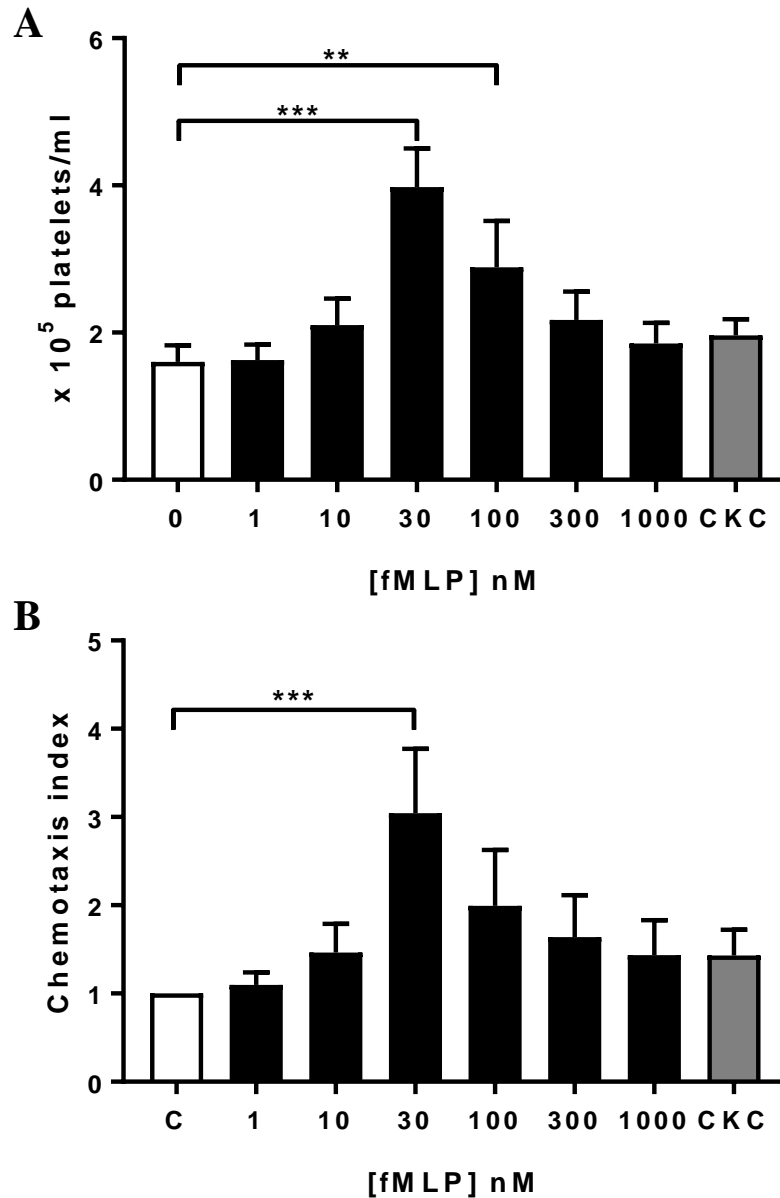


Figure 4.5: fMLP concentration response in optimized transwell chemotaxis assay.

Washed platelets were prepared from citrated human blood. A range of fMLP concentrations were added to the bottom chamber, before the transwell insert was placed into the bottom chamber, and platelets treated with CaCl_2 (2 mM) and ADP (0.1 μM), subsequently added to the insert. The whole chamber was then incubated for 45 minutes. A chemokinetic control group (CKC) had equimolar concentrations of fMLP (30 nM) in top and bottom wells. Following incubation, the number of platelets in the bottom well was enumerated. Data are plotted as the number of platelets/ml (A) and as a chemotaxis index (B). Data are expressed as mean \pm SEM, $n = 10$, $** = P < 0.01$ and $*** = P < 0.001$.

4.2.4 An investigation into the effects of eotaxin, MDC and SDF-1 α on human platelet chemotaxis

CCR3, CCR4 and CXCR4 receptor stimulation produced effects on murine platelet migration in the microplate chemotaxis assay, therefore these chemokines were studied in the optimized human transwell insert chemotaxis assay. The CCR3 receptor chemokine eotaxin caused significant platelet chemotaxis at 30 nM (0 nM vs 30 nM eotaxin: $2.1 \pm 0.3 \times 10^5$ platelets/ml vs $3.6 \pm 0.3 \times 10^5$ platelets/ml, $P < 0.01$) and 100 nM (0 nM vs 100 nM eotaxin: $2.1 \pm 0.3 \times 10^5$ platelets/ml vs $5.4 \pm 0.4 \times 10^5$ platelets/ml, $P < 0.001$) (Figure 4.6A). Similarly, when expressed as a chemotaxis index 30 nM (chemotaxis index: control vs. 30 nM eotaxin: 1.0 ± 0.0 vs. 1.8 ± 0.3 , $P < 0.05$) and 100 nM (chemotaxis index: control vs. 100 nM eotaxin: 1.0 ± 0.0 vs. 2.8 ± 0.3 , $P < 0.001$) eotaxin caused significant platelet chemotaxis (Figure 4.6B).

30 nM and 100 nM MDC also caused significant platelet chemotaxis when expressed as platelets/ml (0 nM vs 30 nM MDC: $2.1 \pm 0.3 \times 10^5$ platelets/ml vs $4.1 \pm 0.5 \times 10^5$ platelets/ml, $P < 0.01$) (0 nM vs 100 nM MDC: $2.1 \pm 0.3 \times 10^5$ platelets/ml vs $5.6 \pm 0.6 \times 10^5$ platelets/ml, $P < 0.001$) (Figure 4.6C) and as a chemotaxis index (chemotaxis index: control vs. 30 nM MDC: 1.0 ± 0.0 vs. 2.1 ± 0.3 , $P < 0.05$) (chemotaxis index: control vs. 100 nM MDC: 1.0 ± 0.0 vs. 2.9 ± 0.5 , $P < 0.001$) (Figure 4.5D), presumably via MDC mediated CCR4 receptor activation. CXCR4 receptor activation via SDF-1 α also caused significant platelet chemotaxis compared to control at 30 nM (0 nM vs 30 nM SDF-1 α : $2.1 \pm 0.3 \times 10^5$ platelets/ml vs $4.4 \pm 0.3 \times 10^5$ platelets/ml, $P < 0.001$) and 100 nM (0 nM vs 100 nM SDF-1 α : $2.1 \pm 0.3 \times 10^5$ platelets/ml vs $5.5 \pm 0.3 \times 10^5$ platelets/ml, $P < 0.001$) (Figure 4.6E). Likewise, when plotted as a chemotaxis index 30 nM (chemotaxis index: control vs. 30 nM SDF-1 α : 1.0 ± 0.0 vs. 2.2 ± 0.3 , $P < 0.01$) and 100 nM (chemotaxis index: control vs. 100 nM SDF-1 α : 1.0 ± 0.0 vs. 2.8 ± 0.3 , $P < 0.001$) SDF-1 α caused significant platelet chemotaxis (Figure 4.6F).

These results demonstrate that human and murine platelets are able to undergo chemotaxis via CCR3, CCR4 and CXCR4 receptor activation *in-vitro*. CCR3 and CCR4

receptor mediated platelet migration are novel findings, with CXCR4 receptor data supporting previous literature (Kraemer et al., 2010).

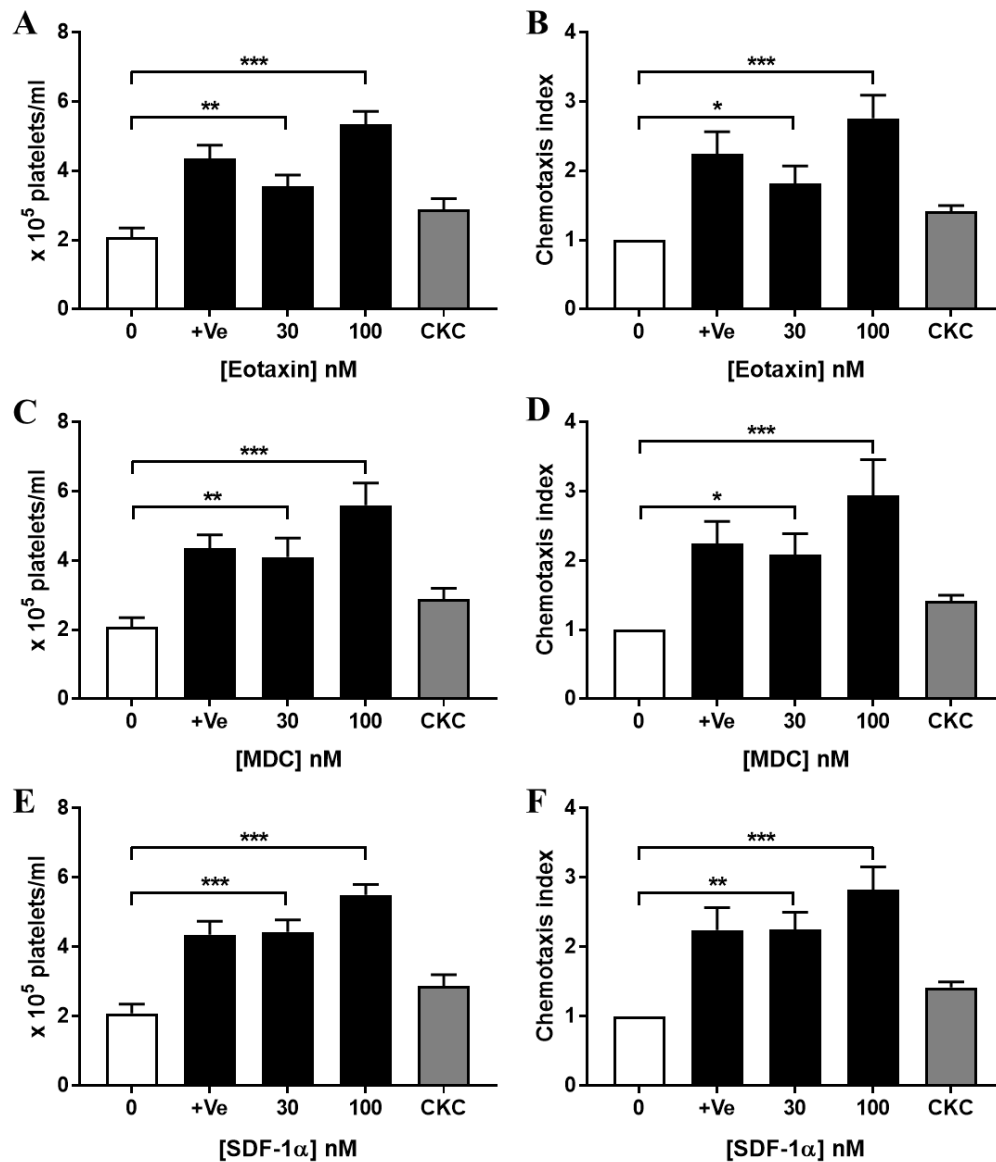


Figure 4.6: The effects of eotaxin, MDC and SDF-1α on human platelet chemotaxis.

Washed platelets were prepared from citrated human blood. A range of eotaxin, MDC and SDF-1α concentrations were added to the bottom chamber, before the transwell insert was placed into the bottom chamber, and platelets treated with CaCl₂ (2 mM) and ADP (0.1 μM), subsequently added to the insert. 30 nM fMLP (+Ve) was used as a positive control. A chemokinetic control group (CKC) had equimolar concentrations of fMLP (30 nM) in top and bottom wells. Following a 45 minute incubation, the number of platelets in the bottom wells was enumerated. Responses to eotaxin (A and B), MDC (C and D) and SDF-1α (E and F) were plotted as the number of platelets/ml and as a chemotaxis index. Data are expressed as mean ± SEM, n = 6, * = P < 0.05, ** = P < 0.01 and *** = P < 0.001.

4.3 Microfluidic chemotaxis assay characterisation studies

4.3.1 Ibidi microslide preparation

The microplate chemotaxis assay, and the transwell chemotaxis assay are high throughput methods for assessing cellular chemotaxis *in-vitro*, however important parameters of chemotaxis such as velocity, direction and the total distance covered by platelets (or leukocytes), cannot be studied in these assay systems. Microfluidic chambers offer more deterministic models that allow a deeper insight into the manner through which cell migration occurs, by providing information on the directionality, velocity and total distance covered by migrating cells. The Ibidi μ -Slide VI^{0.4} microfluidic chamber (microfluidic chemotaxis assay) was chosen as various parameters of chemotaxis can be measured, and due to the assays flexibility in adjusting volumes and coatings specifically for platelet migration. The microfluidic chemotaxis assay was therefore characterized for platelet chemotaxis studies, to enable the visualisation of single platelet migration. These studies were conducted under the supervision of Dr Heidi Welch (Babraham, Institute, Cambridge), whose laboratory have expertise in using microfluidic chambers for the study of neutrophil motility (Johnsson et al., 2014).

IbiTreat (standard surface) microfluidic chemotaxis assay slides were incubated with 10% mouse serum before washing. Then, 50 μ l of washed platelets (1×10^7 /ml) was applied and the platelets allowed time to settle on the surface of the slide. Beads were added to both chemokine and buffer (control) solutions, before their addition at one end of the well and the equivalent volume drawn through the other end. The beads nearest the centre of the well were used as an indicator of the initial distance the chemokine had travelled. Platelet migration toward the chemokine stimulus was recorded using an inverted light microscope, at 12 frames per minute for 8.33 minutes, producing 100 frames. 10-12 platelets were selected at random to be tracked over this time course (Figure 4.7). X and Y coordinates from individual platelet start points were recorded and used to assess platelet migration.

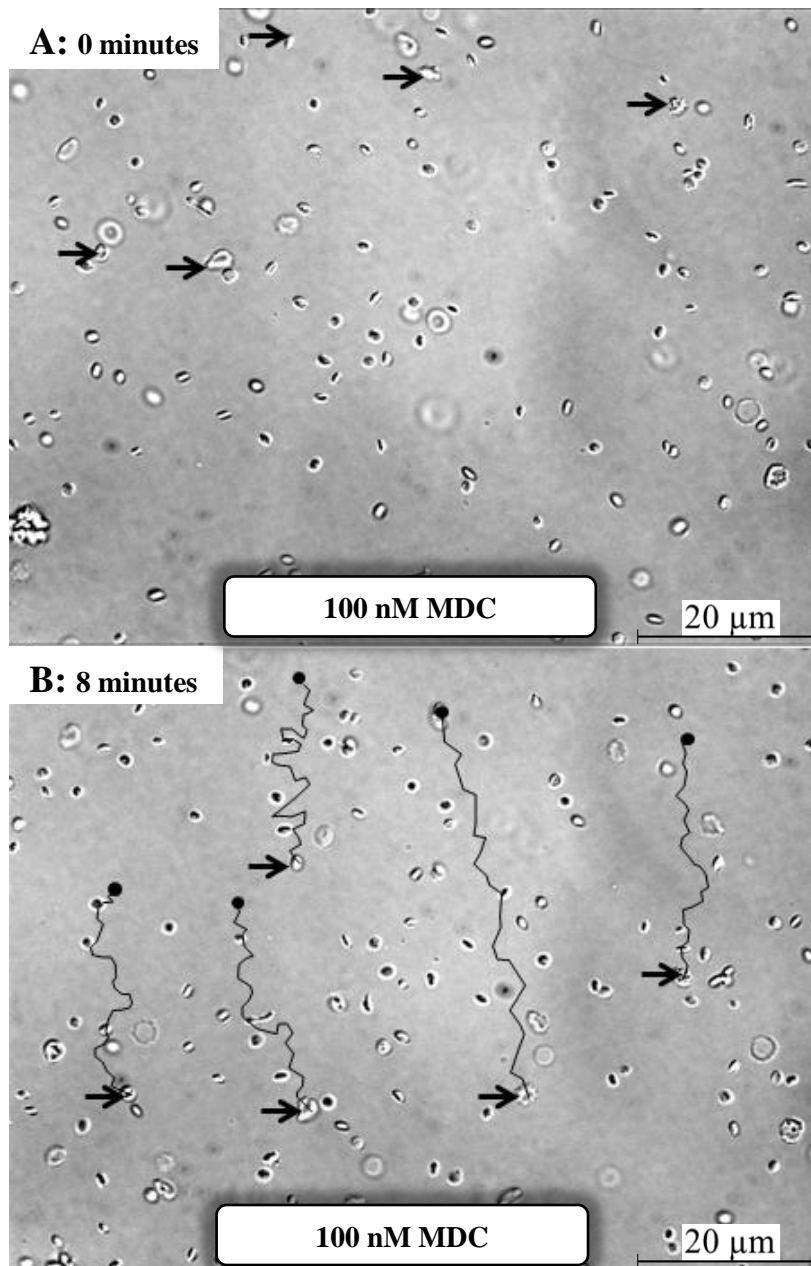


Figure 4.7: Example of platelet tracking in the microfluidic chemotaxis assay.

Washed platelets taken from wild type mice were applied to the microfluidic chemotaxis assay well, followed by MDC (100 nM) applied at one end of the well. In the images presented migration is expected southerly toward MDC. Image A was taken at 0 minutes and image B after 8 minutes. 5 platelets were tracked over this duration, with start points displayed in image A and tracks with end points displayed in image B.

4.3.2 Microfluidic chemotaxis assay pilot study

In model characterisation studies, MDC (100nM) was used to induce platelet chemotaxis, due to its effectiveness to induce platelet chemotaxis taken from naïve mice in the microplate chemotaxis assay. 10% mouse serum coating was applied to the slide for 30 minutes, the slide was then rinsed through and followed by the addition of wild type mouse (WT) washed platelets. The platelets were given 15 minutes to settle on the surface of the slide, then chemokine or control was added and recordings made after 5 minutes. Figure 4.8 displays spider plots of platelets from control and 100 nM MDC treated wells, with individual platelet tracks and average centre of mass (blue dot) presented. Table 3 displays the various measurements of platelet motility that can be created using the microfluidic chemotaxis assay.

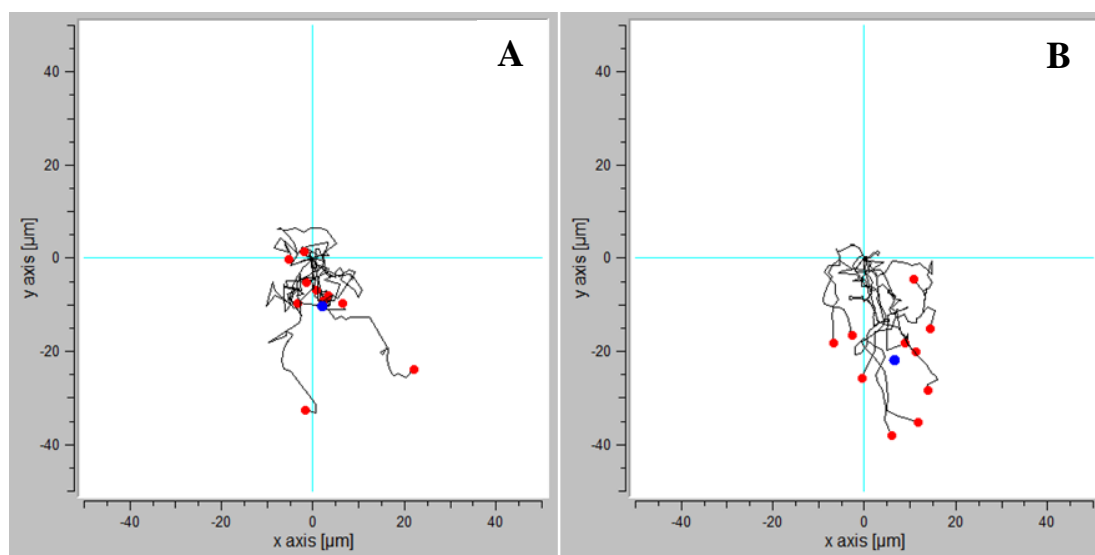


Figure 4.8: Microfluidic chemotaxis assay pilot study spider plots.

Washed platelets taken from wild type mice were added to 10% mouse serum coated microfluidic chambers. The addition of control (A) compared to 100 nM of MDC (B) at one end of the chamber was compared. 10 individual platelets were tracked in each instance. Migration is expected southerly from the origin toward MDC. Red dots show the final location of platelets after tracking from the centre start point. The blue dots represent the average centre of mass. Distance was recorded in μm. Images were taken at 12 frames/minute for 8.33 minutes.

Directionality, which specifies if the movement of platelets was random or toward a general direction, was increased in MDC (100 nM) treated wells (Table 3). Mean accumulated

distance (MAD) of platelets takes into consideration the total distance covered by platelets. There were only slight differences in the MAD, although the mean Euclidean distance (MED), which is the ‘straight line’ distance between the start and end points of platelets, displayed differences between control and MDC (100 nM) treated wells. The speed platelets migrated in the well was similar in both control and MDC treated wells (Table 3).

Table 3: Measurements of platelet motility from pilot microfluidic chemotaxis assay study.

| | Centre of mass (μm) | Directionality | MAD (\pm SEM) (μm) | MED (\pm SEM) (μm) | Mean velocity (\pm SEM) ($\mu\text{m}/\text{sec}$) |
|------------|----------------------------------|----------------|------------------------------------|------------------------------------|---|
| Control | X: 1.80 Y: -10.51 | 0.26 | 45.19 \pm 14.59 | 12.45 \pm 10.87 | 0.37 \pm 0.12 |
| 100 nM MDC | X: 7.083 Y: -21.362 | 0.547 | 44.08 \pm 5.47 | 24.37 \pm 8.72 | 0.36 \pm 0.04 |

The microfluidic chemotaxis assay pilot study revealed single platelet chemotaxis and displayed the usefulness of this assay in providing more information on platelet motility, as differences in the centre of mass, directionality and MED were quantified, and could be assessed visually. Thus, characterisation studies of the microfluidic chemotaxis assay were subsequently performed, to elucidate how the conditions can be altered to optimize the assay.

4.3.3 *Effects of time for platelets to settle on the microfluidic chemotaxis assay slide*

The length of time platelets settled in the microfluidic chemotaxis assay slide, was investigated to help refine the model. Following a 30 minute coating with 10% mouse serum, platelets were left to settle on slides for varied amounts of time before the addition of buffer (control) or chemokine. The centre of mass Y coordinate was used as an indicator of the extent to which platelet chemotaxis had occurred from the origin toward the chemokine stimulus.

Leaving the platelets for 37 minutes to settle caused the greatest platelet chemotaxis (35.8 μm), along with the highest platelet motility measurements (Table 4). 18 minutes was also an effective time period (28.4 μm). After 60 minutes and 105 minutes, platelet chemotaxis

reduced (Figure 4.8). Based on these findings, 30 minutes was selected as the time period for platelets to settle in the microfluidic chemotaxis assay.

Table 4: Measurements of platelet motility from platelet settling time study.

| | Centre of mass (μm) | Directionality | MAD (\pm SEM) (μm) | MED (\pm SEM) (μm) | Mean velocity (\pm SEM) ($\mu\text{m}/\text{sec}$) |
|----------------|----------------------------------|----------------|------------------------------------|------------------------------------|---|
| 30 min Control | X: -5.72 Y: -13.43 | 0.21 | 80.34 ± 10.47 | 17.06 ± 10.50 | 0.32 ± 0.04 |
| 18 min MDC | X: -5.73 Y: -28.40 | 0.35 | 82.37 ± 30.34 | 30.09 ± 15.43 | 0.34 ± 0.12 |
| 37 min MDC | X: -3.00 Y: -35.84 | 0.42 | 86.50 ± 16.61 | 37.57 ± 18.49 | 0.35 ± 0.07 |
| 60 min MDC | X: 6.65 Y: -24.91 | 0.34 | 77.01 ± 24.12 | 27.54 ± 24.77 | 0.31 ± 0.10 |
| 105 min MDC | X: 0.28 Y: -20.48 | 0.37 | 66.33 ± 18.36 | 24.32 ± 10.35 | 0.27 ± 0.07 |

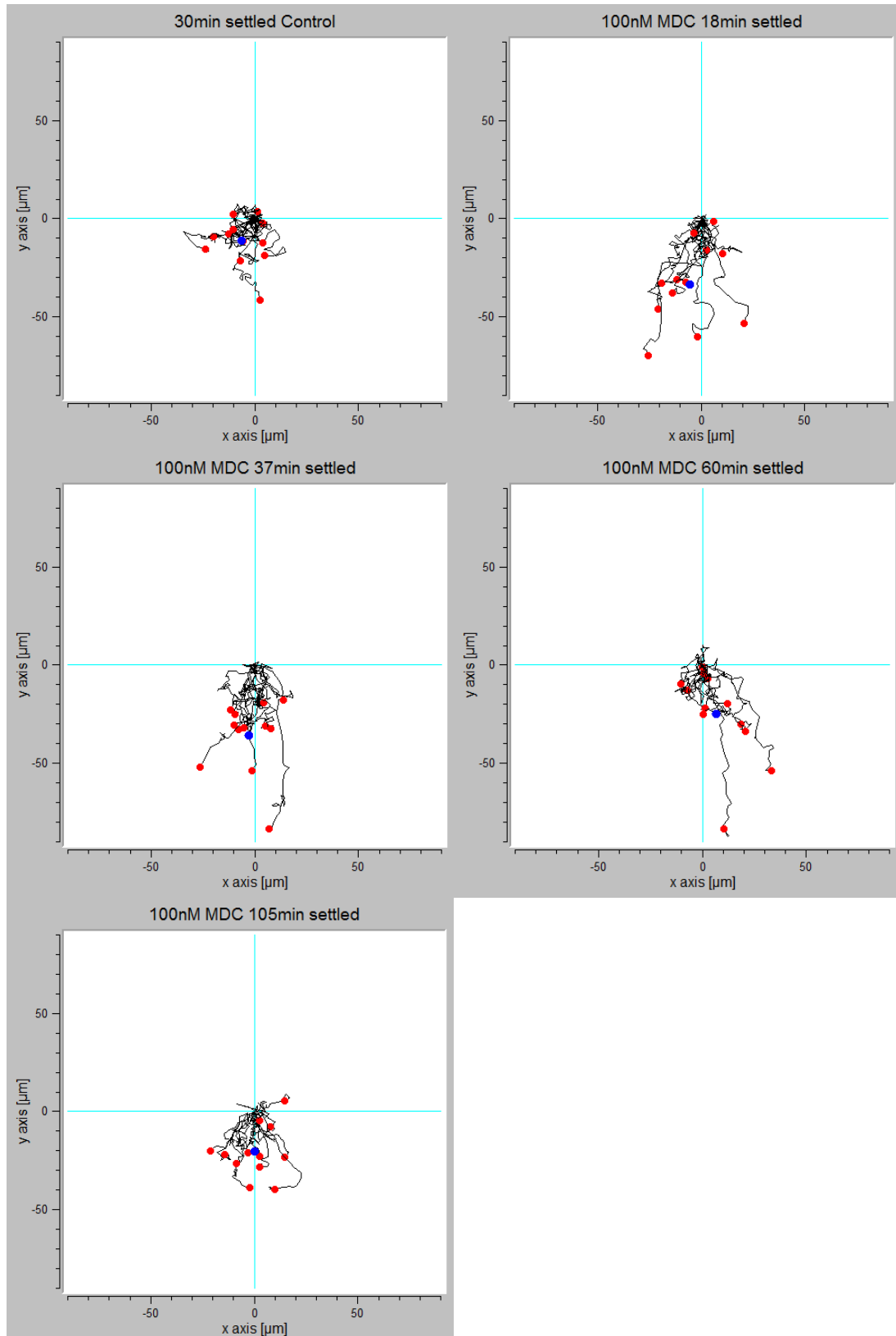


Figure 4.8: The effects of increased platelet settling time on platelet chemotaxis.

Washed platelets taken from wild type mice were added to 10% mouse serum coated microfluidic chambers. In the control sample, platelet chemotaxis was measured following 30 minutes settling time, to control. Platelet chemotaxis following 18, 37, 60 and 105 minutes settling time to MDC (100 nM) was recorded. Migration is expected southerly from the origin. 12 individual platelets were tracked in each instance. Distance was recorded in μm . Images were taken at 12 frames/minute for 8.33 minutes.

4.3.4 Concentration gradient viability study

After chemokine is added to the well, a concentration gradient is formed before diffusion of chemokine and consequently a loss of gradient. The time the concentration gradient is viable after application of MDC (100 nM) to WT washed platelets was investigated. The centre of mass Y coordinate was used as an indicator of platelet chemotaxis. Chemotactic responses persisted for up to 55 minutes (15.2 μm), indicating the concentration gradient was viable long after the addition of chemokine to the slides (Figure 4.9 and Table 5). This allows for recordings to be made over a longer time period as the concentration gradient would be maintained. For microfluidic chemotaxis assay validation studies, platelet migration was recorded 5 minutes following the addition of chemokine.

Table 5: Measurements of platelet motility from concentration gradient viability study.

| | Centre of mass (μm) | Directionality | MAD (\pm SEM) (μm) | MED (\pm SEM) (μm) | Mean velocity (\pm SEM) ($\mu\text{m}/\text{sec}$) |
|------------|----------------------------------|----------------|------------------------------------|------------------------------------|---|
| 5 min MDC | X: 2.52 Y: -5.57 | 0.26 | 40.41 ± 6.69 | 10.86 ± 8.00 | 0.29 ± 0.05 |
| 30 min MDC | X: 1.58 Y: -7.60 | 0.29 | 38.97 ± 6.52 | 11.22 ± 5.04 | 0.27 ± 0.04 |
| 55 min MDC | X: 3.22 Y: -15.21 | 0.38 | 42.93 ± 11.06 | 16.85 ± 11.52 | 0.30 ± 0.08 |

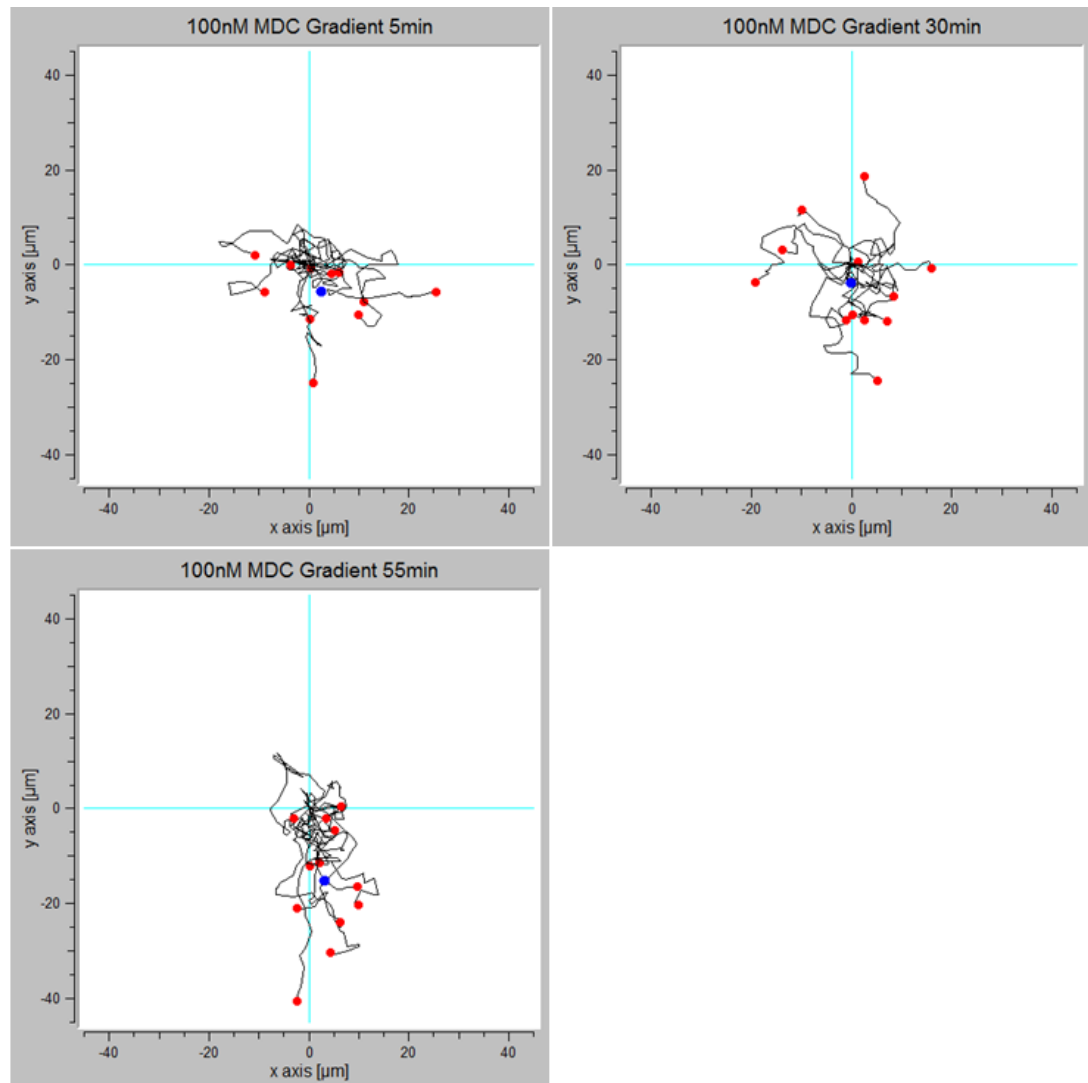


Figure 4.9: The effect of time on the concentration gradient viability in the microfluidic chemotaxis assay.

Washed platelets taken from a wild type mouse were added to a 10% mouse serum coated microfluidic chamber. In the same microfluidic chemotaxis assay chamber, recordings were made after 5, 30 and 55 minutes, following the addition of MDC (100 nM) to washed platelets. Migration is expected southerly from the origin toward MDC. 12 individual platelets were tracked in each instance. Distance was recorded in μm . Images were taken at 12 frames/minute for 8.33 minutes.

4.3.5 Effect of increasing concentrations of MDC and SDF-1 α

To uncover the optimum concentration of MDC for microplate chemotaxis assay validation studies, platelet chemotaxis was investigated to a range of concentrations of MDC. SDF-1 α was also investigated because it has been reported to cause platelet chemotaxis *in-vitro* (Kraemer et al., 2010). Washed platelets were applied to 10% mouse serum coated

microfluidic chambers and given 30 minutes to settle on the surface of the slide. Concentrations of 1 nM, 10 nM, 100 nM and 1000 nM were selected and compared to the addition of buffer (control). 1000 nM of MDC caused the greatest effect on platelet chemotaxis taken from WT mice (25.4 μm) (Figure 4.10), and also had the highest directionality and MED (Table 6). SDF-1 α appeared to cause platelet chemotaxis, but this was not to as great an extent as MDC, with 1000 nM causing the greatest effect (Figure 4.11, Table 7). This subtle SDF-1 α effect on platelet chemotaxis may explain the difficulty in detecting responses in the microplate chemotaxis assay.

Table 6: Measurements of platelet motility in concentration response to MDC study.

| | Centre of mass (μm) | Directionality | MAD (\pm SEM) (μm) | MED (\pm SEM) (μm) | Mean velocity (\pm SEM) ($\mu\text{m}/\text{sec}$) |
|-------------|----------------------------------|----------------|------------------------------------|------------------------------------|---|
| Control | X: -1.02 Y: -6.01 | 0.14 | 56.23 \pm 15.79 | 8.50 \pm 8.63 | 0.23 \pm 0.06 |
| 1 nM MDC | X: 2.29 Y: -16.70 | 0.33 | 58.52 \pm 12.35 | 20.06 \pm 11.81 | 0.24 \pm 0.05 |
| 10 nM MDC | X: -1.48 Y: -13.17 | 0.28 | 58.12 \pm 20.45 | 16.93 \pm 10.98 | 0.24 \pm 0.08 |
| 100 nM MDC | X: 0.91 Y: -12.20 | 0.31 | 52.15 \pm 16.92 | 16.09 \pm 8.68 | 0.21 \pm 0.07 |
| 1000 nM MDC | X: 0.66 Y: -24.29 | 0.45 | 55.37 \pm 11.05 | 25.49 \pm 14.59 | 0.23 \pm 0.05 |

Table 7: Measurements of platelet motility in concentration response to SDF-1 α study.

| | Centre of mass (μm) | Directionality | MAD (\pm SEM) (μm) | MED (\pm SEM) (μm) | Mean velocity (\pm SEM) ($\mu\text{m}/\text{sec}$) |
|------------------------|----------------------------------|----------------|------------------------------------|------------------------------------|---|
| Control | X: -0.64 Y: 0.16 | 0.23 | 48.08 \pm 18.27 | 11.13 \pm 8.81 | 0.20 \pm 0.07 |
| 1 nM SDF-1 α | X: -2.21 Y: -7.75 | 0.24 | 45.34 \pm 16.50 | 12.13 \pm 10.48 | 0.19 \pm 0.07 |
| 10 nM SDF-1 α | X: -0.71 Y: -10.80 | 0.30 | 45.52 \pm 20.29 | 13.84 \pm 10.84 | 0.19 \pm 0.08 |
| 100 nM SDF-1 α | X: 5.39 Y: -6.94 | 0.27 | 49.31 \pm 25.09 | 14.75 \pm 11.89 | 0.20 \pm 0.10 |
| 1000 nM SDF-1 α | X: 5.18 Y: -14.00 | 0.34 | 45.16 \pm 21.83 | 17.44 \pm 16.52 | 0.18 \pm 0.09 |

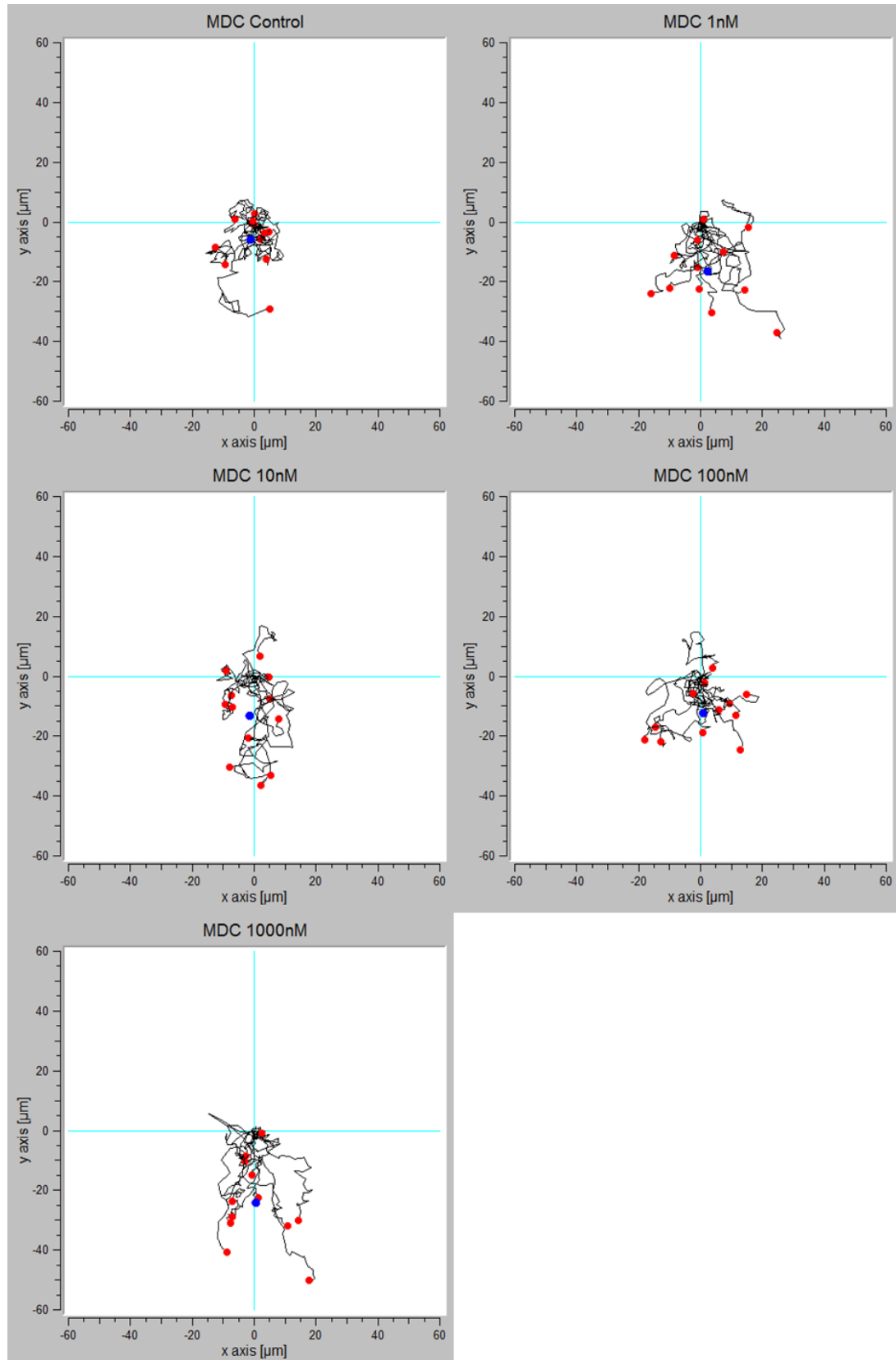


Figure 4.10: Concentration response to MDC in the microfluidic chemotaxis assay.

Washed platelets taken from wild type mice were added to 10% mouse serum coated microfluidic chambers, and allowed 30 minutes to settle on the surface of the well. Washed platelet chemotaxis to a range of MDC concentrations was measured. Migration is expected southerly from the origin toward MDC. 12 individual platelets are tracked in each instance. Distance was recorded in μm . Images were taken at 12 frames/minute for 8.33 minutes.

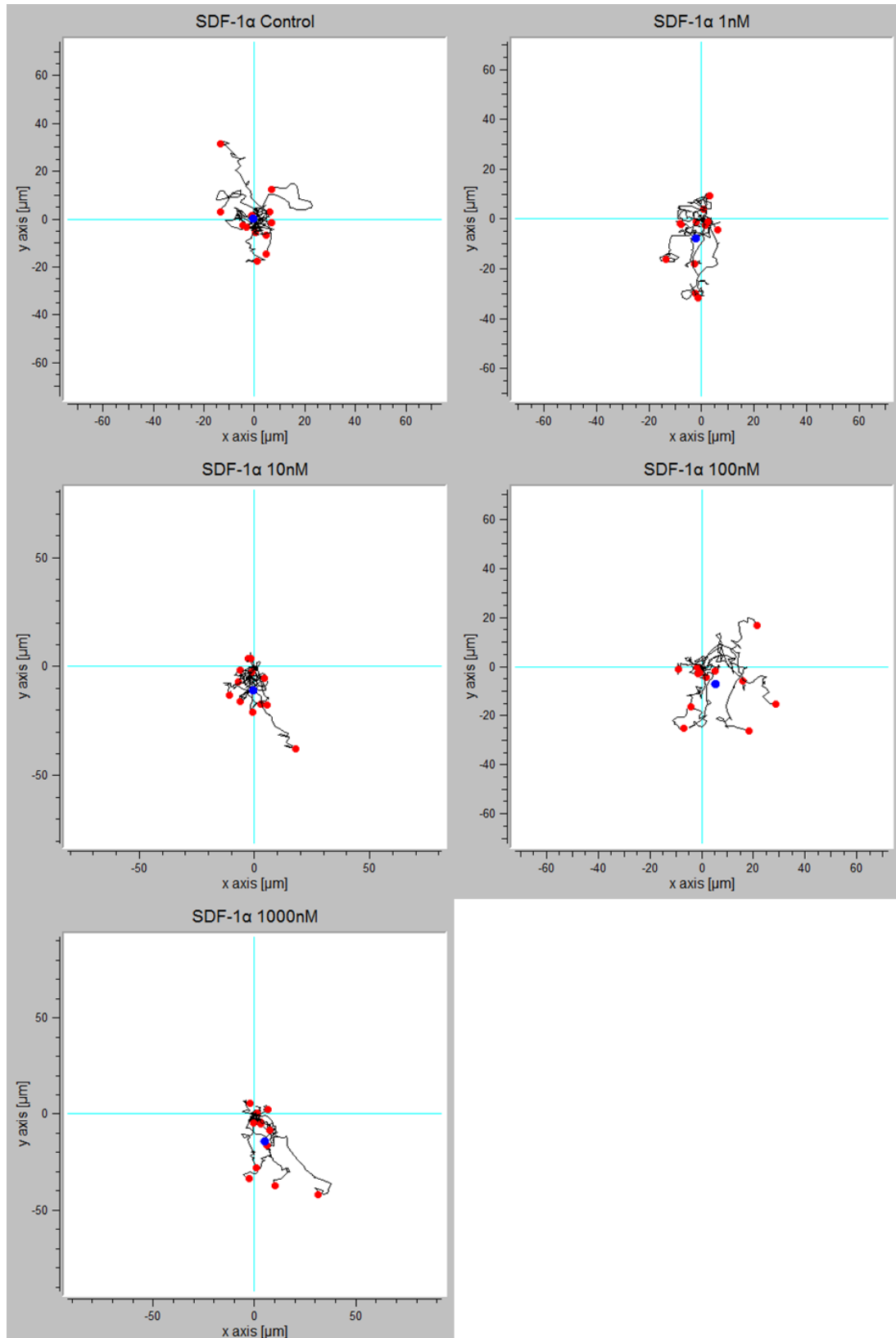


Figure 4.11: Concentrations response to SDF-1α in the microfluidic chemotaxis assay.

Washed platelets taken from wild type mice were added to 10% mouse serum coated microfluidic chambers and allowed 30 minutes to settle on the surface of the well. Washed platelet chemotaxis to a range of SDF-1α concentrations was measured. Migration is expected southerly from the origin toward SDF-1α. 12 individual platelets are tracked in each instance. Distance was recorded in μm. Images were taken at 12 frames/minute for 8.33 minutes.

4.3.6 Platelet firm adhesion to microfluidic chemotaxis assay slide

When moving and adapting the microfluidic chemotaxis assay to a new site (i.e. from the Babraham Institute to KCL), issues regarding the coating of the slides arose. Following a 30 minute incubation with 10% mouse serum, slides were washed and washed platelets added. Platelets are left to settle on the surface of the slide for 30 minutes at which point the platelets become loosely adhered to the surface and chemokine is added. When the methodology was

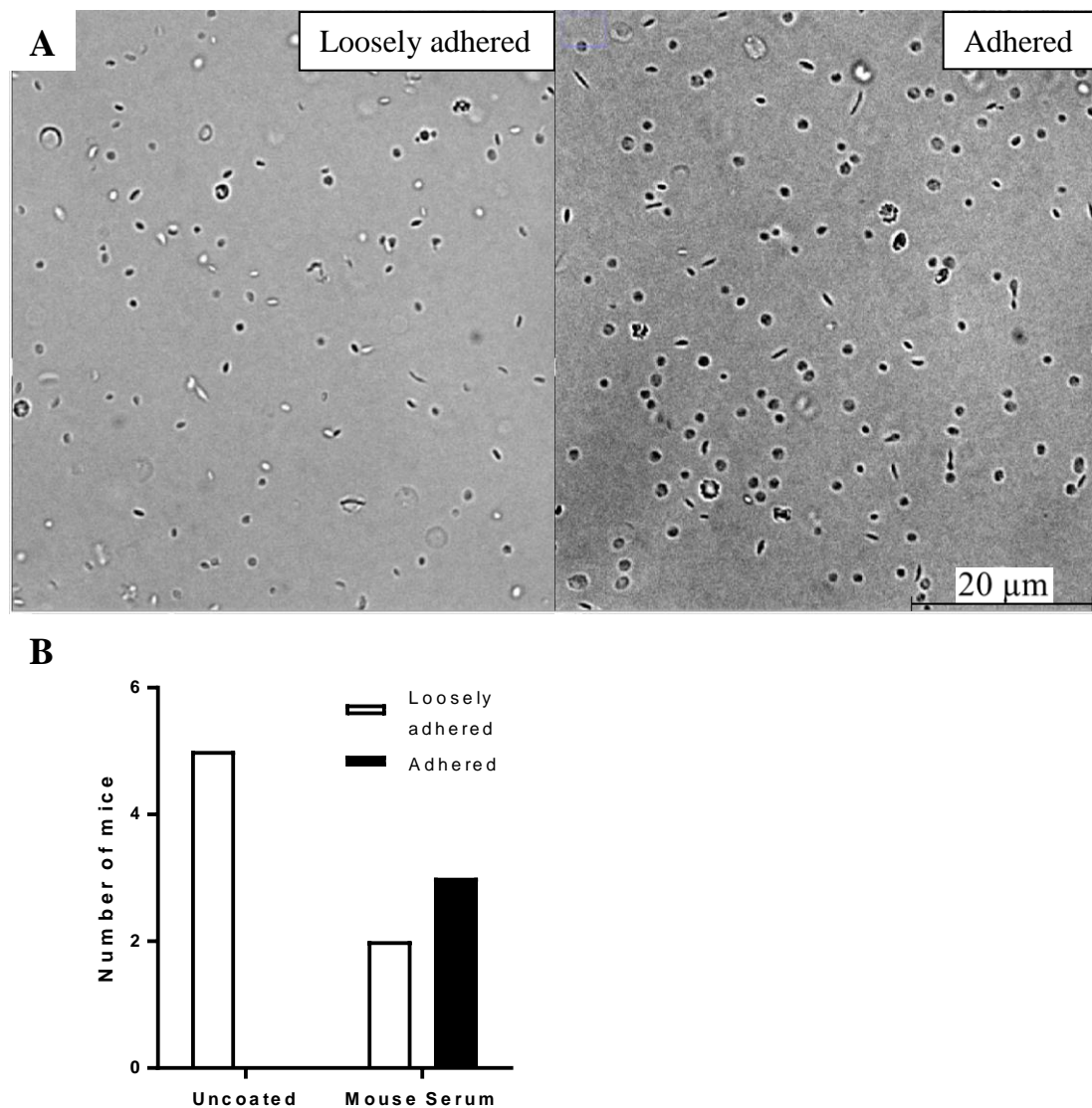


Figure 4.13: Platelet adhesion to the surface of the microfluidic chemotaxis assay slide. A- representative images of platelets loosely adhered and adhered to the microfluidic chemotaxis assay slide surface. B- washed platelets taken from wild type mice were applied to uncoated and 10% mouse serum coated microfluidic chemotaxis assay slides. Adhered was classified as when over 90% of platelets were firmly adhered to the slide. When less than 90% of platelets were firmly adhered, it was classified as loosely adhered. $n = 5$.

transferred to KCL, platelets tended to firmly adhere upon addition to the slide, preventing any movement in response to chemokine (Figure 4.13A).

To investigate if this effect was coating or platelet dependant, washed platelets were added to uncoated and 10% mouse serum coated slides. Platelets were classified as adhered in instances when over 90% in each viewing window remained stationary for the duration of a 8.33 minute video. Otherwise the well was classified as loosely adhered, which typically had 0-5% firmly adhered platelets. Platelets were loosely adhered to uncoated slides, but when coated with mouse serum, platelets became firmly adhered in the majority of wells (Figure 4.13B). This indicated that 10% mouse serum was not a reliable coating solution for the microfluidic chemotaxis assay.

4.3.7 Testing different well coatings for platelet chemotaxis

A more reproducible coating for the microfluidic chemotaxis assay was sought after issues with 10% mouse serum coating. A range of coating solutions were tested using 100 nM MDC to induce platelet chemotaxis. Pre-treated Ibidi collagen IV and Ibidi fibronectin coated slides were tested, along with uncoated, 10% mouse serum, 10 µg/ml vitronectin and 10 µg/ml fibrinogen coated slides. Coating solution was added to microfluidic chemotaxis assay slides for 30 minutes and then washed. Washed platelets were then applied and allowed 30 minutes to settle on the surface of the well, before the addition of control or MDC (100 nM). After 5 minutes, platelet migration was recorded and videos analysed using the centre of mass Y coordinate as an indicator of platelet chemotaxis.

Coating slides with either mouse serum, collagen, fibronectin or vitronectin did not influence platelet migration towards MDC (100 nM) compared to control (0 nM MDC) (Figure 4.14). However, platelets that were added to slides with 10 µg/ml fibrinogen coating revealed a difference between control and MDC treated wells (control 0 nM vs. MDC 100 nM : $15.9 \pm 2.5 \mu\text{m}$ vs. $27.2 \pm 4.5 \mu\text{m}$). It is noted that Kraemer and colleagues also used fibrinogen

to coat wells when performing platelet chemotaxis to SDF-1 α , therefore 10 μ g/ml fibrinogen was used moving forward (Kraemer et al., 2010).

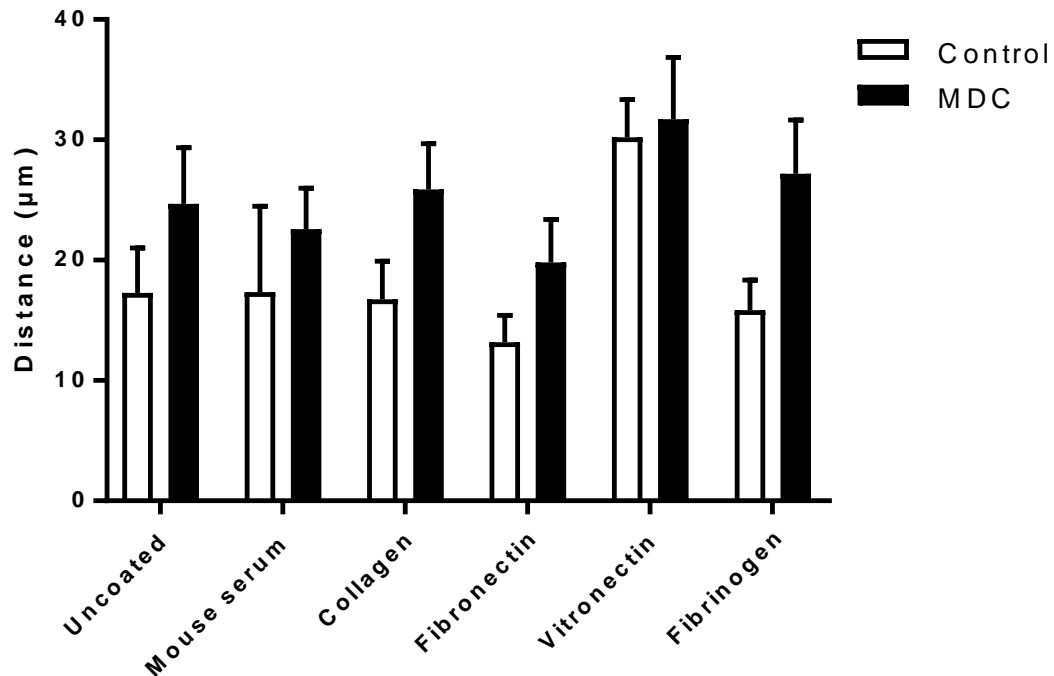


Figure 4.14: The effects of different well coatings on platelet chemotaxis.

Washed platelets chemotaxis to control and MDC (100 nM) was performed on pre-treated Ibidi collagen IV and Ibidi fibronectin coated slides and on uncoated, 10% mouse serum, 10 μ g/ml vitronectin and 10 μ g/ml fibrinogen coated slides in the microfluidic chemotaxis assay. 12 individual platelets were tracked in each instance. The centre of mass Y coordinate, from the start position was recorded in μ m. Data presented as mean \pm SEM and n = 4.

4.3.8 Elucidation of the basal movement of platelets in control groups

Before moving forward with the assay, the basal effect of adding control to the microfluidic chemotaxis assay well was investigated, because it was noted in all studies that a basal movement of platelets occurred, and methodological enhancements to reduce this effect might make the assay more precise when measuring cellular chemotaxis across small distances by increasing the measurement ratio between control and chemokine-stimulated groups. Therefore, multiple elements of the assay were separated to determine their effects on the basal movement platelets.

After 30 minutes of settling time, recordings of platelets before any further addition to the well (unstimulated platelets) were undertaken, which had minimal effects on the movement of platelets (Figure 4.15). Similarly, when platelets were incubated with 2 mM calcium chloride before addition to the well, minimal effects on the movement of platelets occurred, indicating that stimulation with 2 mM calcium chloride does not contribute to the basal movement of platelets by enhancing platelet activation due to experimental procedure and handling *per se*.

When comparing the addition of control to unstimulated platelets, there was a significantly greater movement of platelets in the Y coordinate direction toward control (unstimulated vs. control: $1.5 \pm 0.8 \mu\text{m}$ vs. $17.9 \pm 0.9 \mu\text{m}$, $P < 0.01$) (Figure 4.15). This

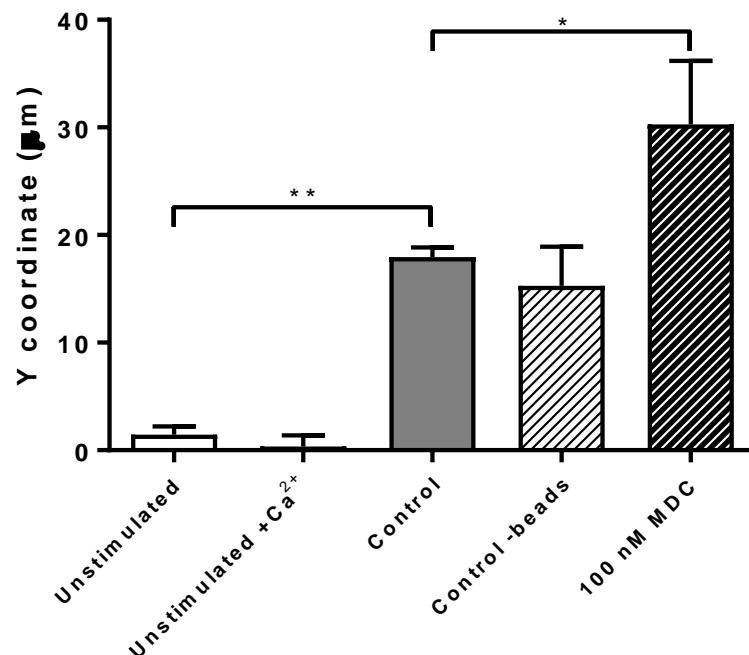


Figure 4.15: Elucidation of control drifting effect.

Washed platelets were taken from wild type mice. Unstimulated and unstimulated platelets incubated with calcium, were applied directly to $10 \mu\text{g/ml}$ fibrinogen coated microfluidic chemotaxis assay wells and recordings made after 30 minutes. Control, control -beads or MDC (100 nM) was applied at one end of the microfluidic chemotaxis assay well containing unstimulated washed platelets. 12 individual platelets were tracked in each instance. The centre of mass Y coordinate, from the start position was recorded in μm . Data presented as mean \pm SEM, $n = 2-4$, * = $P < 0.05$ and ** = $P < 0.01$.

suggests that the addition of fluid (control) is causing a drifting flow effect of platelets toward the side of the well where the fluid (control) was injected.

The potential effects of beads on the movement of platelets was investigated, as beads are added along with assay diluent (control) and chemokine in the microplate chemotaxis assay. Control assay diluent without beads (control-beads) caused a similar effect on the movement of platelets as control, indicating the addition of beads were not the cause of the movement of platelets in control samples. MDC (100 nM) stimulation caused a significant increase in platelet chemotaxis compared to control in fibrinogen coated slides (control vs. 100 nM MDC: $17.9 \pm 0.9 \mu\text{m}$ v.s. $30.3 \pm 5.9 \mu\text{m}$, $P < 0.05$), demonstrating the ability of the assay to induce platelet chemotaxis, irrespective of platelet drifting effects.

The underlying cause of this platelet drifting phenomena remain unknown. The microfluidic chemotaxis assay has the potential to be an effective model of platelet chemotaxis and has been used here to provide quantifiable visual evidence of platelet motility towards a chemotactic gradient, as measured by Euclidian distance, and directionality. Cellular velocity was not modulated by chemokine stimulation of platelets, suggesting that random movement of platelets occurs extensively in this assay. The optimal conditions for platelet migration in the microfluidic chemotaxis assay requires further investigation before the effects of allergen sensitization on platelet motility can be studied.

**Chapter 5 Results III: Development of a
HDM-sensitization and exposure
model, to assess platelet recruitment,
adhesion and migration in response to
allergen challenge**

5.1 HDM allergen-sensitization model validation

5.1.1 Initial studies investigating the effects of OVA-sensitization on intravital cremaster muscle preparations

In order to produce a model for evaluating the vascular activity of platelets in response to allergen challenge *in-vivo*, intravital microscopy of the mouse cremaster muscle was investigated. The cremaster muscle is a thin piece of muscle tissue that can be dissected and viewed whilst maintaining the blood circulation of the muscle in anaesthetized mice. Due to the thickness of the muscle, clear visualization of the vasculature can be obtained, and with the addition of a fluorescent platelet specific antibody, the activity of platelets can also be studied (Jenne et al., 2011). This is an important consideration, since more pertinent tissues for studying allergic inflammation (e.g. the lung), are inherently more unstable preparations due to their delicate nature, the physical movement due to respiratory manoeuvres, the vital physiological role of the lungs that requires sympathetic surgical techniques, and their enclosed position within the body, making clear visualization of cellular adhesive events difficult to interpret without the use of sophisticated technology and adaptations (Looney et al., 2011; Looney and Bhattacharya, 2014; Rodriguez-Tirado et al., 2016). This is further confounded when trying to focus on smaller cells such as platelets.

Because OVA-sensitization of mice was used successfully for investigations of platelet migration *in-vitro*, using the microplate chemotaxis assay, attempts were made to use the same sensitization protocol for *in-vivo* intravital experiments. However, when using this same OVA-sensitization protocol for preparations of cremaster muscle, an accumulation of white solids within the scrotal sack was observed in both sham and OVA-sensitized mice, that was absent in naïve mice (Figure 5.1A, B and D). Furthermore, the cremaster muscle appeared inflamed with leukocytes extensively covering the muscle tissue (Figure 5.1C). The protocol for both sham and OVA sensitized mice involves the intraperitoneal co-administration of aluminium hydroxide solution as an adjuvant. Thus, the deposits were deduced to be aluminium hydroxide accumulating in the testis, causing localized inflammation. This

inflammation would mask investigatory effects of allergen-induced inflammation, and allergen-induced stimulation of platelets, therefore the OVA-sensitization model was no longer considered.

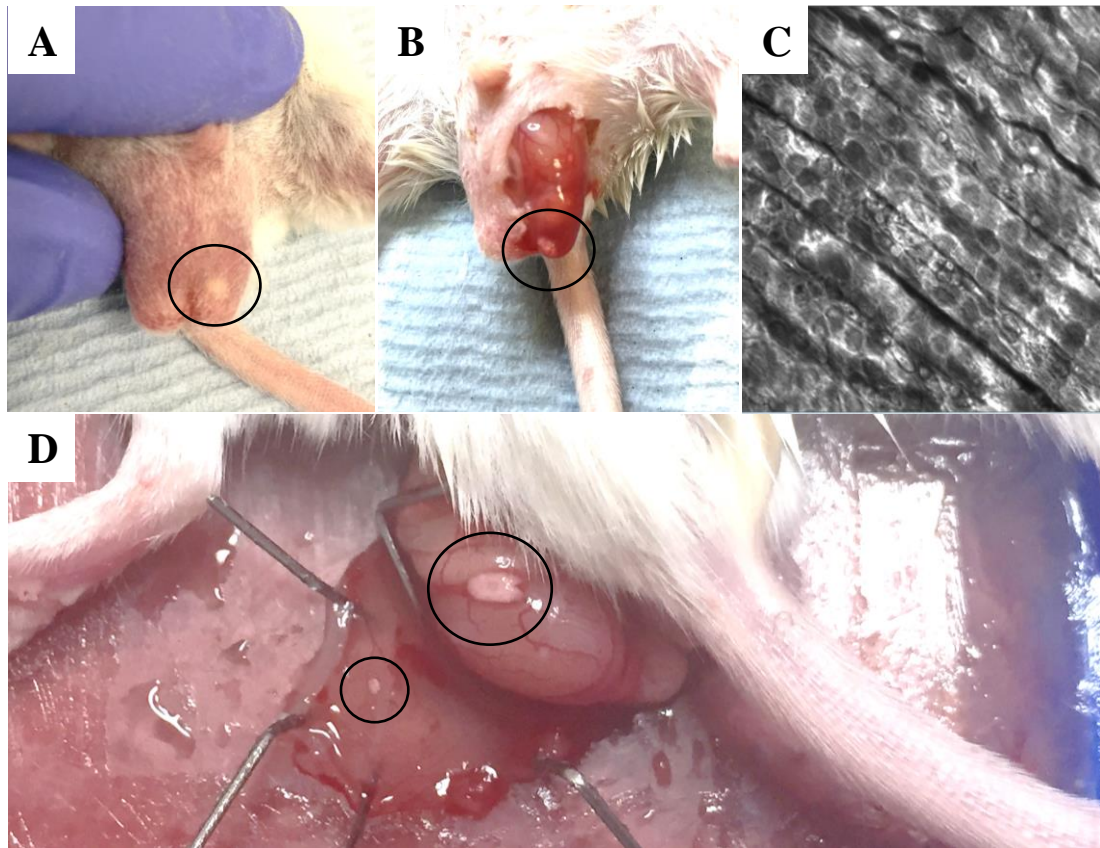


Figure 5.1: Aluminium hydroxide deposits in the scrotum.

Representative images of sham-sensitized mouse cremaster muscle, with black circles highlighting areas of aluminium hydroxide deposits (A, B and D). C- sham-sensitized mouse cremaster muscle, viewed under bright field light, using a 63x objective lens.

5.1.2 HDM-sensitization protocol development

Due to difficulties with the OVA-sensitization protocol, the use of alternative allergens was explored to allow for intravascular platelet activity investigations in response to allergen challenge. One drawback of the OVA model is that humans are not sensitized to OVA, making the model less relevant to that which occurs clinically (Gregory and Lloyd, 2011). As humans can become sensitized to HDM, and due to supporting literature demonstrating the allergic profile in murine models of allergic airways inflammation with pertinence to asthma, a HDM-

sensitization protocol was investigated to enable intravital microscopy studies in response to allergen challenge (Gregory et al., 2009).

A two week HDM-sensitization period (25 µg i.n., 5 days/week) was used because this protocol was previously demonstrated to induce a significant number of eosinophils within the lung and BAL samples of HDM-sensitized mice compared to other time periods (Gregory et al., 2009). On day 13, mice were exposed again to HDM (25 µg i.n.) and 24 hours later BAL samples were taken. Sham-sensitized mice received saline i.n. for two weeks, before subsequent 25 µg HDM exposure on day 13. There was a significant increase in pulmonary leukocyte recruitment of HDM-sensitized mice compared to sham-sensitized mice (sham vs. HDM: $16.1 \pm 1.1 \times 10^4$ cells/ml vs. $32.5 \pm 5.6 \times 10^4$ cells/ml, $P < 0.05$) (Figure 5.2A). The proportion of eosinophils and neutrophils was also elevated in the BAL fluid after HDM-sensitization (sham vs. HDM: $0 \pm 0 \times 10^4$ eosinophils/ml vs. $12.6 \pm 8.0 \times 10^4$ eosinophils/ml, $P < 0.05$ and 0 ± 0 vs. 5.0 ± 2.7 neutrophils, $P < 0.05$) (Figure 5.2B). The increased numbers

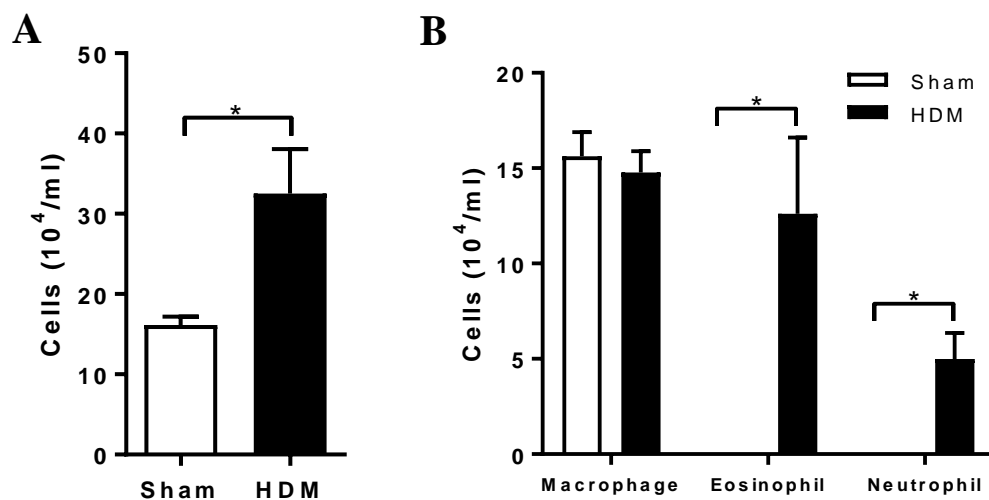


Figure 5.2: The effects of HDM-sensitization on pulmonary leukocyte recruitment.

Sham-sensitized and HDM-sensitized mice (25 µg HDM on days 0, 1, 2, 3, 4, 7, 8, 9, 10, and 11) were challenged with HDM (25 µg i.n.) on day 13, and 24 hours later BAL fluid was collected. A- total BAL leukocytes. B- differential cell counts. Data presented as mean \pm SEM, $n = 4$ and $* = P < 0.05$.

of eosinophil therefore indicate an allergic inflammatory response was generated following a two week sensitization protocol.

5.1.3 Histological staining of eosinophils in lungs of HDM-sensitized mice

The elevated numbers of eosinophils present in the BAL fluid, was supported by histological staining of HDM-sensitized mouse lungs. The Luna stain marks the cytoplasm of eosinophils red, distinguishing them from other leukocytes (Figure 5.3A). The number of eosinophils within the airway walls was elevated in HDM-sensitized mice compared to sham-sensitized mice (sham vs. HDM: 0.1 ± 0.0 eosinophils/mm vs. 12.7 ± 1.7 eosinophils/mm, $P < 0.001$), again suggesting this model is effective at producing an allergic inflammatory response (Figure 5.3B).

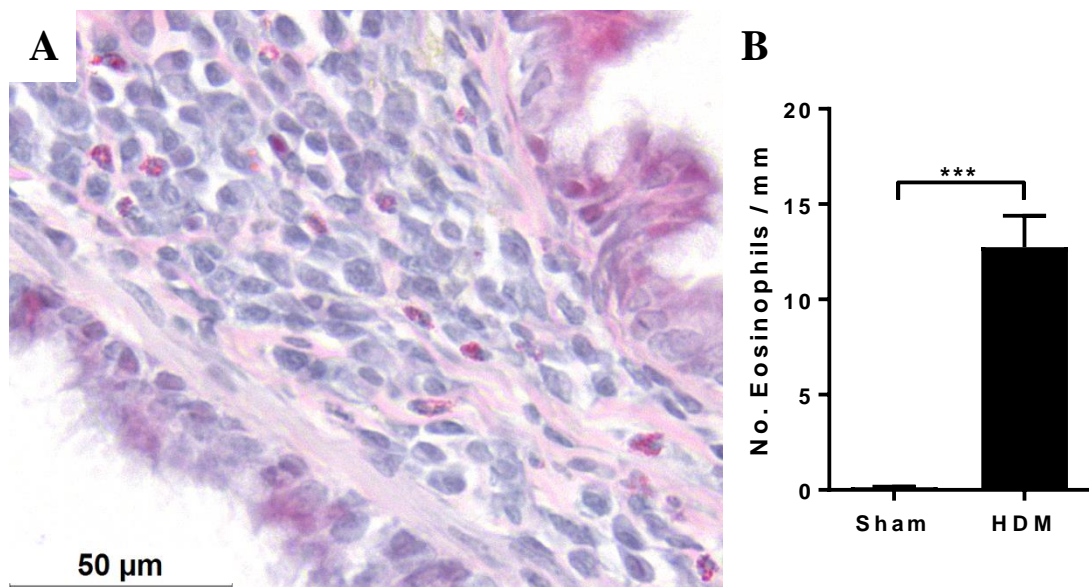


Figure 5.3: The effects of HDM-sensitization on eosinophil recruitment to the airway walls.

Sham-sensitized and HDM-sensitized mice (25 μg HDM on days 0, 1, 2, 3, 4, 7, 8, 9, 10, and 11) were challenged with HDM (25 μg i.n.) on day 13, and 24 hours later the lungs dissected, formalin fixed and paraffin wax embedded. Sections of lung were histologically stained for eosinophils. A- representative image of eosinophils stained red within the airway wall of HDM-sensitized mouse lung. B- the number of eosinophils in the airway wall was quantified and expressed per mm along the airway wall. Data presented as mean \pm SEM, $n = 4$ and *** = $P < 0.001$.

5.1.4 Platelets in extravascular lung compartments of HDM-sensitized mice

The lungs of HDM-sensitized mice were immunohistochemically stained for platelets using an anti-mouse CD42b antibody, specific for platelet recognition (Figure 5.4A). Platelets have previously been shown in extravascular regions of lungs from OVA-sensitized mice in response to OVA-challenge (Pitchford et al., 2008). There was an increased presence of platelets located around the airway walls of HDM-sensitized mice compared with sham-sensitized mice (sham vs. HDM: 0.4 ± 0.2 platelets/mm vs. 3.8 ± 0.4 platelets/mm, $P < 0.001$) (Figure 5.4B), indicating that HDM-sensitization can induce platelet recruitment to the lungs.

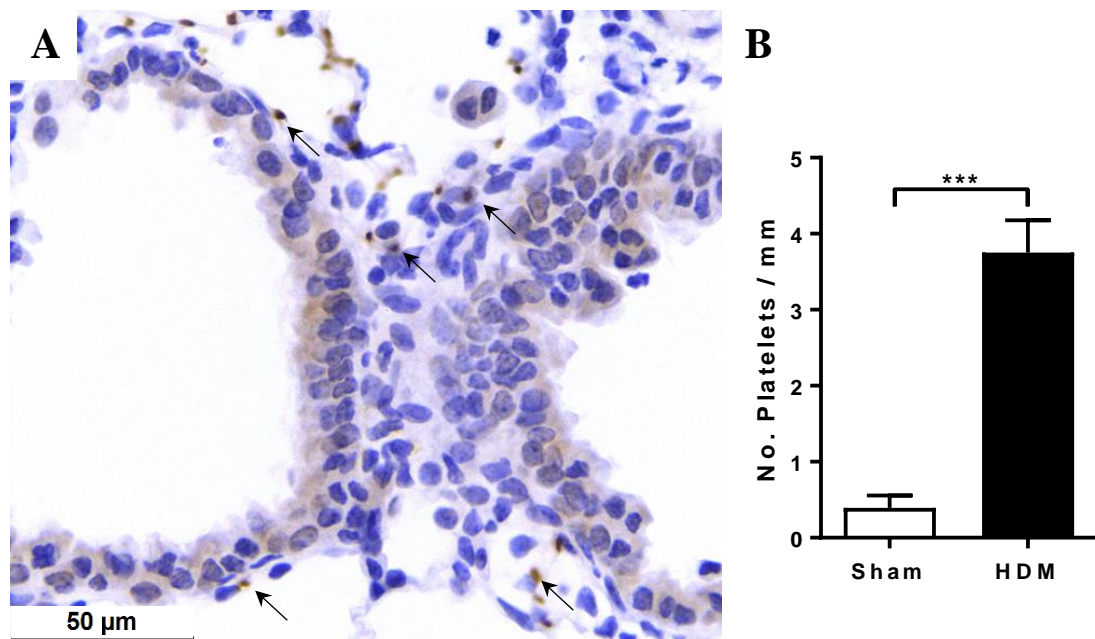


Figure 5.4: The effects of HDM-sensitization on platelet recruitment to the airway walls. Sham-sensitized and HDM-sensitized mice (25 μg HDM on days 0, 1, 2, 3, 4, 7, 8, 9, 10, and 11) were challenged with HDM (25 μg i.n.) on day 13, and 24 hours later the lungs dissected, formalin fixed and paraffin wax embedded. Sections of lung were immunohistochemically stained using platelet specific anti-human CD42b antibody. A- representative image of platelets stained brown within the airway wall of HDM-sensitized mouse lung (highlighted with arrows). B- the number of platelets in the airway wall was quantified and expressed per mm along the airway wall. Data presented as mean ± SEM, n = 4 and *** = $P < 0.001$.

5.1.5 HDM systemic sensitization investigation

HDM-sensitized mice were assessed to investigate if the allergen-sensitization had become systemic and not limited to localization to the lung, since the purpose of the change in allergen model was for use in the cremaster muscle model of intravital microscopy. Proof of the ability of intranasally delivered allergen (HDM) to induce systemic sensitization was investigated by observing oedema formation at an anatomical site distant from the lungs injected with HDM (the shaven skin on the backs of mice), using Evans Blue dye as a marker of extravasated protein (albumin). Sham-sensitized and HDM-sensitized mice were therefore administered Evans blue dye (0.5% in saline, i.v.) 30 minutes before i.d. challenge with 25 μ l saline and 25 μ g/25 μ l HDM on either side of the shaved backs of mice.

In sham-sensitized mice, oedema formation at 15 and 60 minutes following saline and HDM injection was similar, suggesting any oedema formation was the result of localized trauma, rather than the sensitizing agent *per se* (Figure 5.5). In HDM-sensitized mice, oedema formation around the injection site with HDM was visibly more pronounced than with saline injection at 15 minutes and this increased further at 60 minutes after injection with HDM (Figure 5.5). This suggests an allergic inflammatory response was achieved in a distant anatomical region in response to intranasally administered HDM and thus the mice were systemically sensitized to HDM.

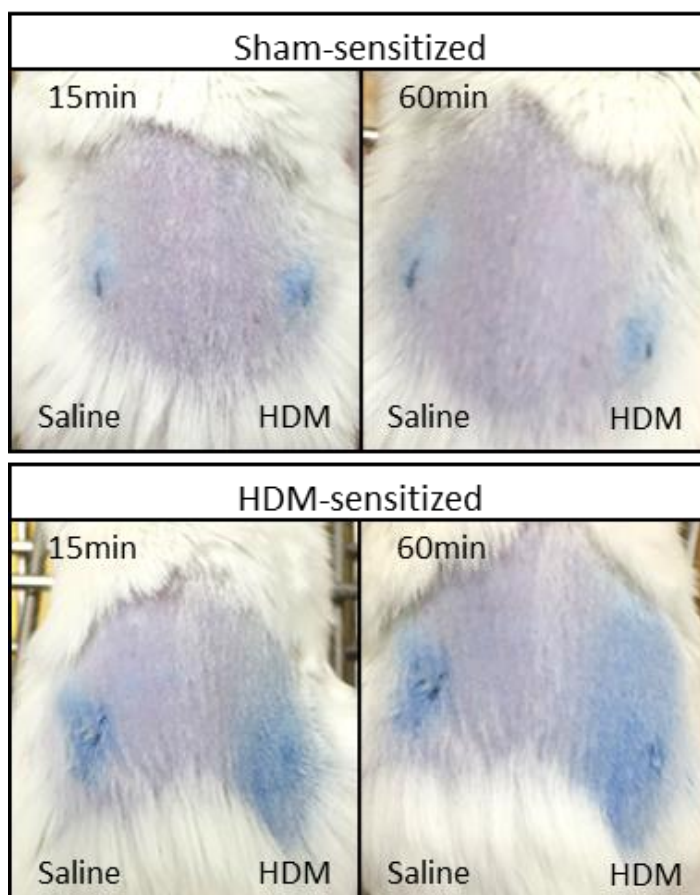


Figure 5.5: The effects allergen exposure on s.c. oedema formation.

Sham-sensitized and HDM-sensitized mice (25 μ g HDM on days 0, 1, 2, 3, 4, 7, 8, 9, 10, and 11) were administered 0.5% Evans blue dye i.v. (0.1 ml) on day 14, and 30 minutes later 25 μ l of saline and 25 μ g/25 μ l of HDM were administered i.d. on the left and right sides of the dorsum, respectively. Images of the dorsum were taken after 15 and 60 minutes on a sham-sensitized and an HDM-sensitized mouse.

5.2 Intravital microscopy protocol development for assessing platelet recruitment in response to allergen challenge

5.2.1 Cremaster muscle imaging set up

Using the optimized HDM-sensitization protocol detailed in section 5.1, intravital microscopy of the mouse cremaster muscle was investigated to study the intravascular activity of platelets in response to allergen challenge. In order to visualise platelets, mice were administered with a platelet specific anti-mouse CD49b PE conjugated antibody i.v., 1 hour before each cremaster muscle dissection (Jenne et al., 2011). Observing the cremaster muscle under bright field light allows the tissue architecture to be clearly observed (Figure 5.6A). When switching to 580nm fluorescent light, the platelets are visible, however the tissue architecture is lost (Figure 5.6B). By dampening the bright field light and applying the fluorescent light, a merged display was created allowing platelets and the architecture of the tissue to be observed (Figure 5.6C). A minimum of three 10 second videos of different post

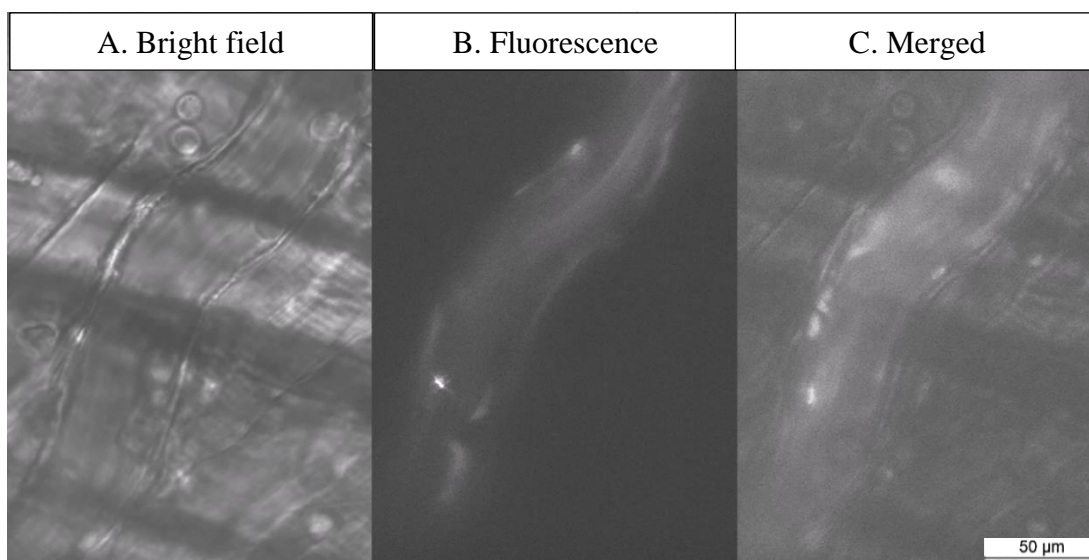


Figure 5.6: Representative images of cremaster muscle in bright field light, 580 nm fluorescence light and merged displays.

A HDM-sensitized mouse (25 µg HDM on days 0, 1, 2, 3, 4, 7, 8, 9, 10, and 11) was challenged with HDM (100 µg s.c. to the scotum) on day 13. 24 hours later anti-mouse CD49b PE-conjugated antibody was administered (i.v.) and the cremaster muscle dissected for intravital microscopy. Representative images of post capillary venules viewed under A- bright field light, B- 580 nm fluorescent light and C- merged displays, using a 63x objective lens.

5.2.2 Allergic inflammatory response in the cremaster muscle

The number of leukocytes in extravascular compartments of cremaster muscle was used as an indicator of the severity of the inflammatory response. Naïve, sham-sensitized and HDM-sensitized mice were challenged with 100 µg HDM subcutaneously (s.c.) on the scrotum or saline s.c., 24 hours before cremaster muscle dissection. A comparison between acute saline and HDM challenge s.c., revealed elevated numbers of extravascular leukocytes in the cremaster muscle of HDM-challenged mice (saline vs. HDM: 2.6 ± 0.9 cells/30 µm² vs. 16.1 ± 2.6 cells/30 µm², $P < 0.001$) (Figure 5.7). This indicated HDM alone causes an elevated inflammatory response in the cremaster muscle, in non allergen-sensitized mice.

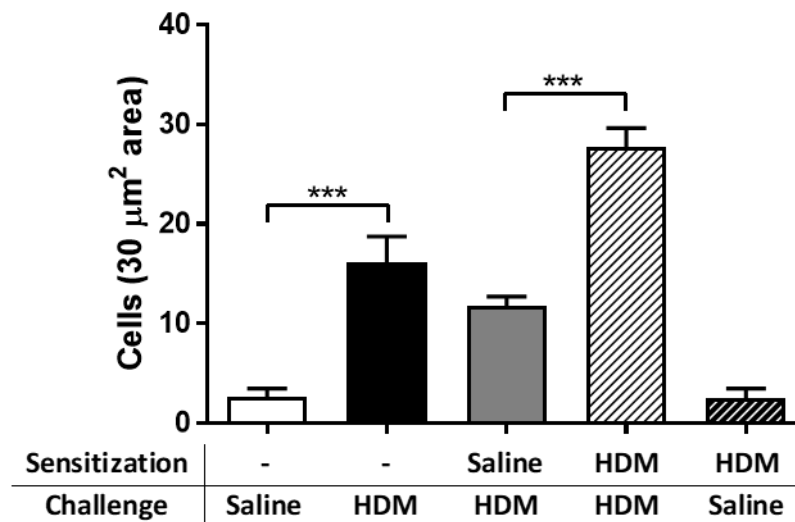


Figure 5.7: The effects of s.c. HDM challenge on leukocyte migration to the cremaster muscle.

Sham, sham-sensitized and HDM-sensitized mice (25 µg HDM on days 0, 1, 2, 3, 4, 7, 8, 9, 10, and 11) were challenged with saline or HDM (100 µg) s.c. on the scrotum on day 13. 24 hours later anti-mouse CD49b PE-conjugated antibody was administered (i.v.), the cremaster muscle was dissected and a minimum of 3 post capillary venules recorded for 10 seconds. The number of leukocytes in extravascular compartments was quantified and expressed per µm². Data presented as mean \pm SEM, $n = 4-7$ and *** = $P < 0.001$.

When mice were HDM-sensitized and challenged with HDM s.c. on the scrotum, a further elevation in the numbers of leukocytes that were present in extravascular compartments of the cremaster muscle in HDM-sensitized mice was observed (saline-

sensitized, HDM challenged vs. HDM-sensitized, HDM challenged: 11.8 ± 0.9 cells/ $30 \mu\text{m}^2$ vs. 27.8 ± 1.9 cells/ $30 \mu\text{m}^2$, $P < 0.001$). The increased inflammatory response in HDM-sensitized mice suggested that an allergic inflammatory response was generated using this model. A final HDM-sensitized and saline challenged group was included to act as an additional control, showing comparable responses to acute saline control group.

5.2.3 *Eosinophil staining of cremaster muscle*

Following intravital microscopy experiments, the cremaster muscle was processed and stained for eosinophils via histological analysis. The Luna staining technique stains the cytoplasm of eosinophils red, distinguishing them from other cell types (Figure 5.8A). The number of eosinophils in extravascular compartments of cremaster muscle was compared between groups. In HDM-sensitized and HDM-challenged mice, the number of eosinophils was significantly elevated compared to sham-sensitized HDM-challenged mice (saline-sensitized, HDM challenged vs. HDM-sensitized, HDM challenged: 3.90 ± 1.0 eosinophils/ mm^2 vs. 27.8 ± 7.7 eosinophils/ mm^2 , $P < 0.001$) (Figure 5.8B).

As the recruitment of eosinophils are a requisite feature in allergic inflammatory responses, these data supported the conclusion in section 5.2.2, that HDM challenge in the cremaster muscle of intranasally HDM-sensitized mice was capable of causing an allergic inflammatory response (Calhoun et al., 1991).

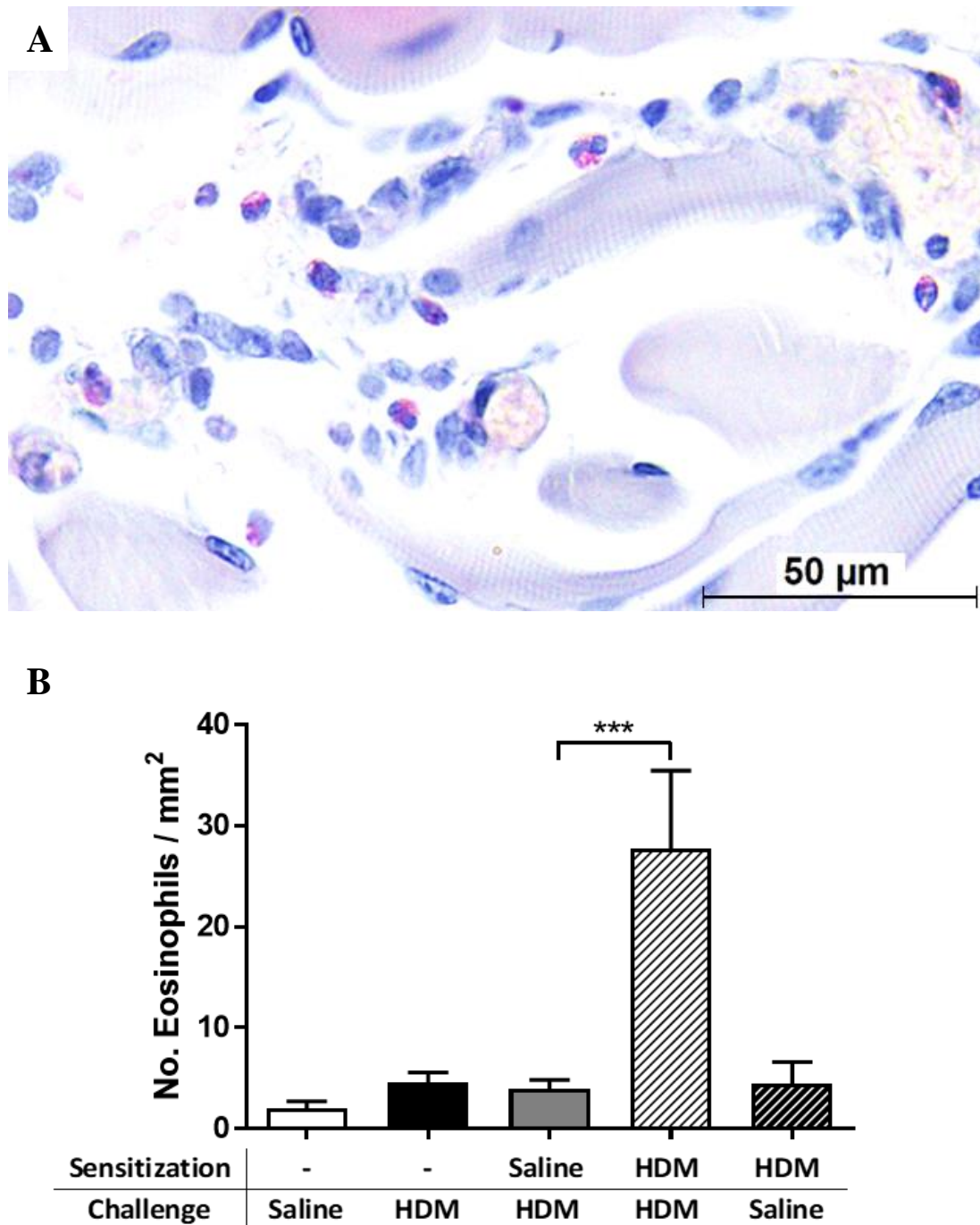


Figure 5.8: The effects of s.c. HDM challenge in HDM-sensitized mice on eosinophil migration to the cremaster muscle.

Sham, sham-sensitized and HDM-sensitized mice (25 μ g HDM on days 0, 1, 2, 3, 4, 7, 8, 9, 10, and 11) were challenged with saline or HDM (100 μ g) s.c. to the scrotum on day 13. 24 hours later the cremaster muscle was formalin fixed and paraffin wax embedded. Sections of cremaster muscle were histologically stained for eosinophils. A- representative image of eosinophils stained red within the cremaster muscle of HDM-sensitized and HDM-challenged mouse. B- the number of eosinophils in extravascular compartments of cremaster muscle was quantified and expressed per mm² of tissue. Data presented as mean \pm SEM, n = 4-7 and *** = P < 0.001.

5.2.4 Platelet activity in response to allergen challenge in the cremaster muscle

The labelling of platelets with anti-mouse CD49b PE conjugated antibody in intravital microscopy preparations of cremaster muscle, allowed for detailed tracking of adhesion events of individual platelets. In response to HDM challenge in the cremaster muscle, distinct single platelet adhesion and rolling events on the endothelium were observed. Due to the dimmed bright field light, leukocytes could also be observed, allowing single platelet events to be distinguished from platelets attached to leukocytes. Platelet adhesion events were classified as instances where a single platelet would remain bound to the endothelium for the duration of a 10 second video recording. Platelet rolling events were classified when single platelets would slow and interact with the wall of the endothelium, and then either remain bound or continue on in the bloodstream. Thus, vascular platelet activity in response to allergen challenge was determined by assessing platelet adhesion and platelet rolling events.

Following 100 µg HDM administration s.c. to the scrotum of healthy mice, an increase in both platelet adhesion and platelet rolling events was recorded compared to saline s.c. (saline challenge vs. HDM challenge: 0.1 ± 0.1 cells/30 µm² vs. 1.3 ± 0.2 cells/30 µm², platelets adhered, $P < 0.01$ and 10.2 ± 1.0 rolling/10 seconds vs. 18.8 ± 1.1 rolling/10 seconds, platelet rolling events, $P < 0.01$), indicating that after an acute inflammatory challenge, an increase in platelet activity occurred. In HDM-sensitized and HDM challenged mice, platelet adhesion and platelet rolling events were further significantly elevated (saline-sensitized, HDM challenged vs. HDM-sensitized, HDM challenged: 1.0 ± 0.2 cells/30 µm² vs. 2.1 ± 0.3 cells/30 µm², platelet adhesion, $P < 0.01$ and 17.9 ± 1.9 rolling/10 seconds vs. 32.1 ± 2.3 rolling/10 seconds, platelet rolling events, $P < 0.001$) (Figure 5.9), indicating allergen challenge caused increased intravascular platelet adhesion to the vasculature. No incidence of intravascular platelet aggregation, or embolus formation was noted in these video recordings.

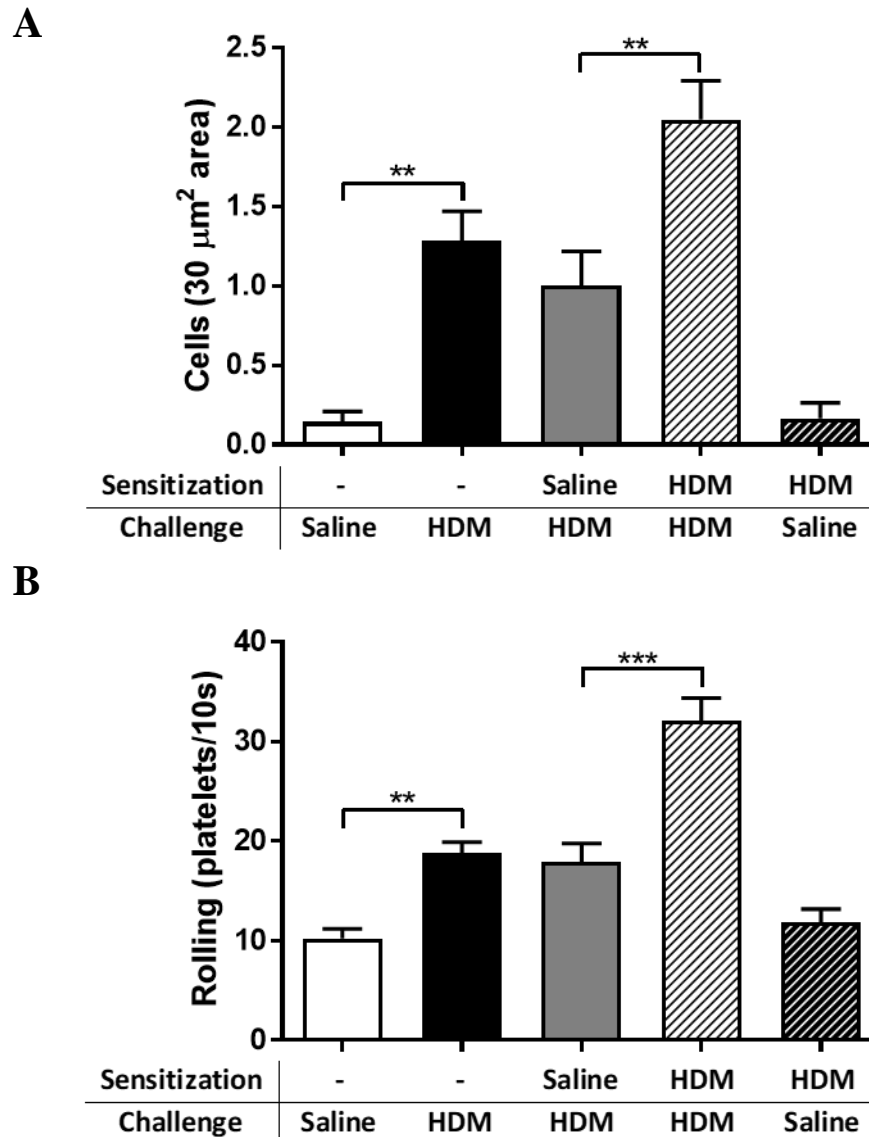


Figure 5.9: The effects of s.c. HDM challenge in HDM-sensitized mice on intravascular platelet adhesion and platelet rolling events.

Sham, sham-sensitized and HDM-sensitized mice (25 μg HDM on days 0, 1, 2, 3, 4, 7, 8, 9, 10, and 11) were challenged with saline or HDM (100 μg) s.c. to the scrotum on day 13. 24 hours later anti-mouse CD49b PE-conjugated antibody was administered (i.v.), the cremaster muscle was dissected and a minimum of 3 post capillary venules recorded for 10 seconds. A- the number of platelet adhesion events was quantified and expressed per μm^2 . B- the number of platelet rolling events during each 10 second video was quantified. Data presented as mean \pm SEM, $n = 4-7$, $** = P < 0.01$ and $*** = P < 0.001$.

5.2.5 Platelet migration to cremaster muscle following allergen challenge

Following intravital microscopy experiments described in section 5.2.4, the cremaster muscle was harvested and processed for immunohistochemical staining, using a platelet

specific anti-mouse CD42b antibody (Amison et al., 2018b). The number of platelet specific, brown staining events in extravascular portions of cremaster muscle was quantified and compared between groups (Figure 5.10).

The numbers of platelets in extravascular regions of cremaster muscle tended to increase following acute HDM challenge (1.5 ± 0.4 platelets/mm² vs. 5.0 ± 1.5 platelets/mm²). The number of migrated platelets in the HDM-sensitized and HDM-challenged group increased compared with the sham-sensitized HDM-challenged group (sham-sensitized, HDM challenged vs. HDM-sensitized, HDM challenged: 4.7 ± 1.4 platelets/mm² vs 11.7 ± 2.4 platelets/mm², $P < 0.01$). These data showed that an allergic inflammatory response resulted in the accumulation of platelets in the cremaster muscle.

Mice were successfully sensitized to HDM allergen and upon exposure to allergen in the cremaster muscle, an allergic inflammatory response was generated. Single platelet rolling and adhesion events on the endothelium were visualized within the cremaster muscle vasculature *in-vivo*, and such events were increased following allergen exposure. This allergic inflammatory response of platelets was supported by immunohistochemical data showing platelet accumulation in extravascular compartments of the cremaster muscle following allergen exposure, which is likely preceded by distinct platelet rolling and adhesion events. The intravital microscopy model was optimized for platelet investigations, and will allow the mechanisms by which platelets respond in allergic inflammatory conditions *in-vivo*, to be explored.

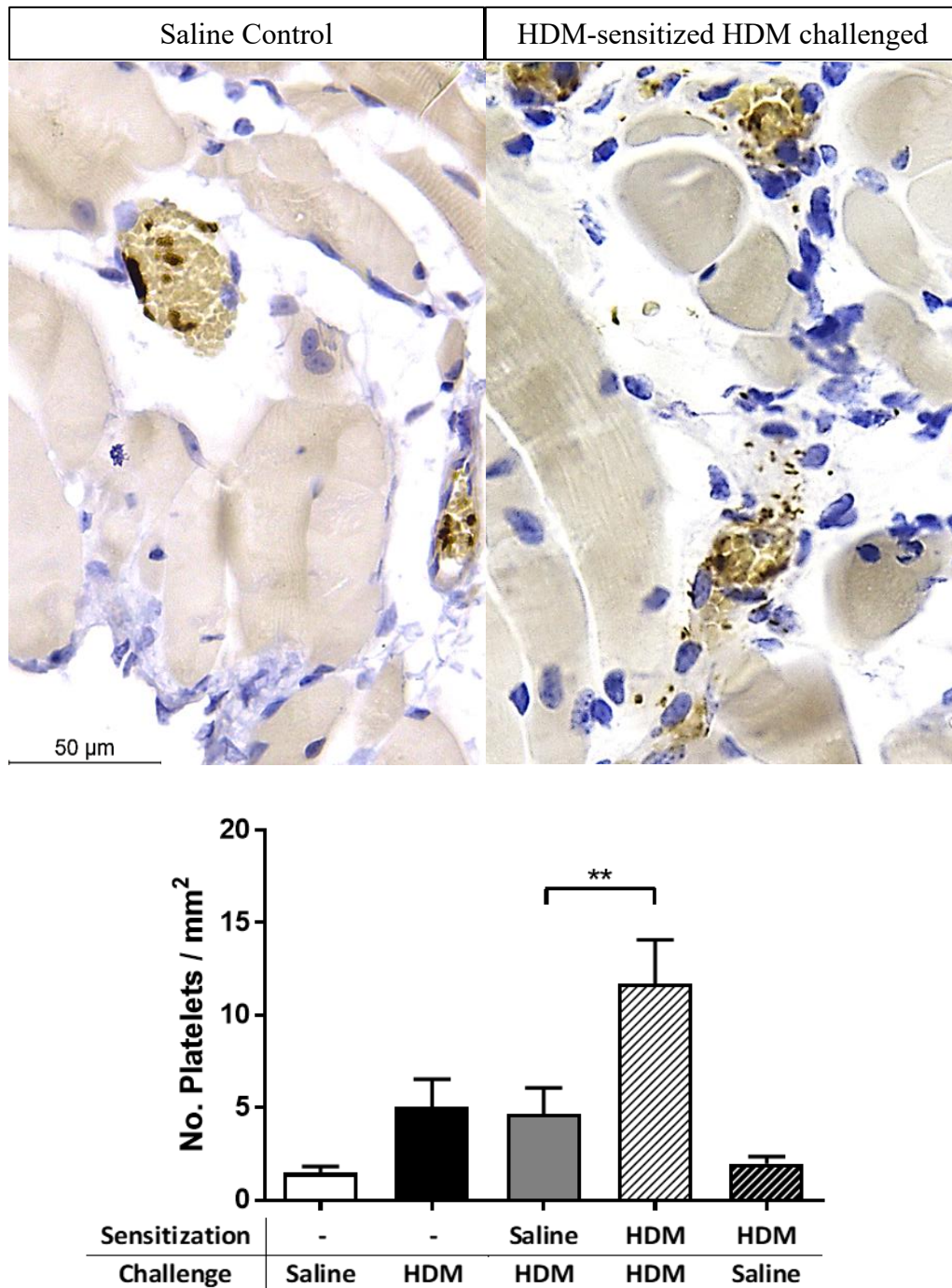


Figure 5.10: The effects of HDM-sensitization and HDM challenge on platelet migration to the cremaster muscle.

Sham, sham-sensitized and HDM-sensitized mice (25 µg HDM on days 0, 1, 2, 3, 4, 7, 8, 9, 10, and 11) were challenged with saline or HDM (100 µg) s.c. on the scrotum on day 13. 24 hours later the cremaster muscle was formalin fixed and paraffin wax embedded. Sections of cremaster muscle were immunohistochemically stained using platelet specific anti-mouse CD42b antibody. Representative images of platelets stained brown in saline control and HDM-sensitized HDM challenged mice are presented. The number of platelets in extravascular compartments of cremaster muscle was quantified and expressed per mm² of tissue. Data presented as mean ± SEM, n = 4-7 and ** = P < 0.01.

**Chapter 6 Results IV: The roles of
chemokine receptors on platelet tissue
recruitment and accumulation in
response to allergen challenge**

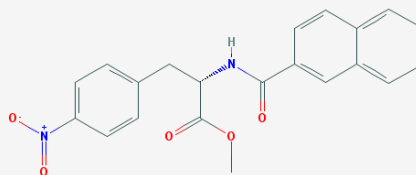
6.1 The effects of platelet chemokine receptor antagonists in the lung

6.1.1 Effect of chemokine receptor antagonists on platelet migration following allergen challenge

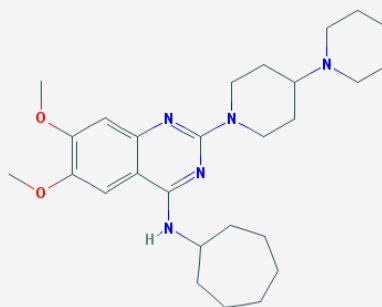
The mechanisms which underlie platelet migration to sites of allergic inflammation are unknown, but data suggests a potential role of platelet chemokine receptors in this process. Platelet chemokine receptor expression was shown through flow cytometry and western blot analysis, with subtle differences in expression levels following allergen-sensitization. Murine and human platelet chemotaxis was observed *in-vitro* to chemokine receptor ligands, demonstrating the functional activity of platelet chemokine receptors. This activity was increased when murine platelets were harvested from allergen-sensitized mice. An allergic inflammatory model to study platelet activity *in-vivo* showed increased platelet rolling and adhesion to the vasculature, and the increased extravascular accumulation of platelets in allergen-sensitized mice subsequently exposed to allergen. Building on these findings, chapter 6 explores the roles of platelet chemokine receptors on *in-vivo* platelet recruitment, adhesion and extravascular accumulation in allergen-sensitized mice exposed to allergen, using chemokine receptor antagonists.

SB328437, C-021 and AMD3100 are small molecule antagonists to CCR3, CCR4 and CXCR4 receptors, respectively (Figure 6.1). Following the HDM-sensitization protocol, on day 13 mice were treated with vehicle, 30 mg/kg SB328437, 30 mg/kg C-021 or 10 mg/kg AMD3100 (all i.p.). Mice were then challenged with saline or HDM (100 µg i.n.) and 24 hours later the lungs were harvested and stained via immunohistochemistry using an anti-mouse CD42b platelet specific antibody. In the sham group, mice were sensitized to HDM before administration of vehicle (i.p.) and challenged with saline (i.n.) on day 13, with lungs harvested on day 14.

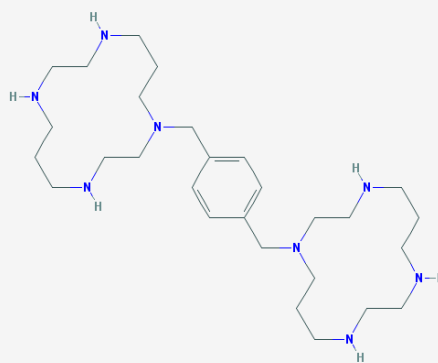
SB328437



C-021



AMD3100

**Figure 6.1: SB328437, C-021 and AMD3100 chemical structures.**

Chemical structure images taken from National Center for Biotechnology Information, PubChem Compound Database; CID=5311092 (SB328437), CID=56972238 (C-021) and CID=129856585 (AMD3100) (accessed Oct. 19, 2018).

The number of platelets in extravascular compartments along the airway walls was quantified and compared between groups using a phase contrast light microscope (Zeiss Axiovert) with a 40x objective lens. There was no significant difference between administering HDM-sensitized mice saline or HDM (100 μg i.n.) (HDM-sensitized, saline-challenged group vs. HDM-sensitized, HDM-challenged group: 2.0 ± 0.3 platelets/mm vs. 3.0

± 0.4 platelets/mm) (Figure 6.2), therefore the effects of chemokine receptor antagonists on platelet migration were difficult to determine.

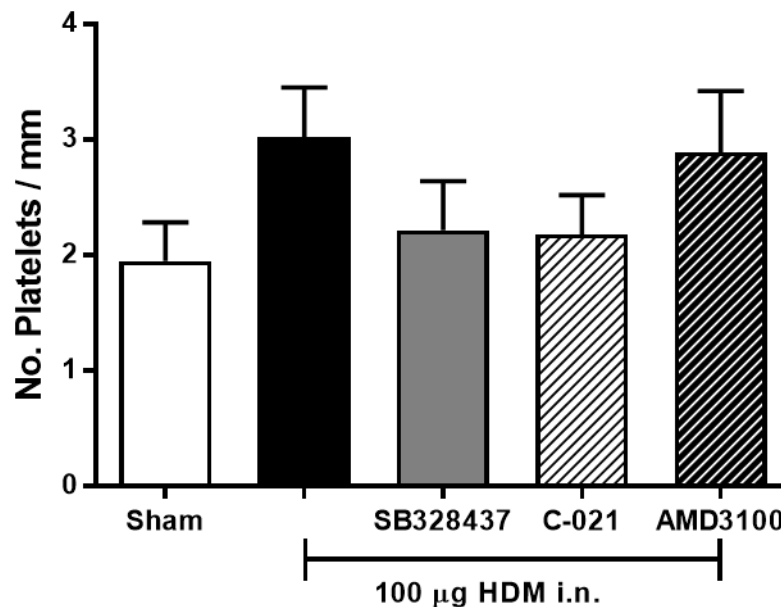


Figure 6.2: The effects of chemokine receptor antagonists on platelet migration following allergen challenge.

HDM-sensitized mice (25 μ g HDM on days 0, 1, 2, 3, 4, 7, 8, 9, 10, and 11) were administered vehicle, SB328437 (30 mg/kg i.p.), C-021 (30 mg/kg i.p.) or AMD3100 (10 mg/kg i.p.) on day 13, 30 minutes before saline or HDM (100 μ g i.n.) challenge. On day 14, the lungs were dissected, formalin fixed and paraffin wax embedded. Sections of lung were immunohistochemically stained using platelet specific anti-mouse CD42b antibody. The number of platelets in the airway wall was quantified and expressed per mm along the airway wall. Data presented as mean \pm SEM and $n = 8$.

6.1.2 The effects of chemokine receptor antagonists following a one week recovery period

Following ten separate i.n. doses of 25 μ g HDM over a two week period, an accumulation of platelets and other inflammatory cells occurs in the lungs as a result of each subsequent HDM-exposure, contributing to both allergen-sensitization and pulmonary recruitment of inflammatory cells (Gregory et al., 2009). It was therefore hypothesised that with HDM-challenge occurring three days (day 13) post the final sensitization (day 10), inflammatory cells within the lung would not have sufficient time to clear, therefore masking the effects of any one particular HDM challenge on leukocyte recruitment, or the influence of

chemokine receptors on this response. In order to overcome this issue, following the final sensitization, mice were given an additional 7 days to recover from the initial 2-week HDM-sensitization period. On day 20 mice were administered chemokine receptor antagonists (vehicle, 30 mg/kg SB328437, 30 mg/kg C-021 or 10 mg/kg AMD3100, all i.p.) and challenged with saline or HDM (100 µg i.n.) and 24 hours later lungs were harvested. In the sham group, mice were sensitized to HDM before administration of vehicle (i.p.) and challenged with saline (i.n.) on day 20, with lungs harvested on day 21.

There was a significant difference in the number of platelets in extravascular compartments of the airway walls between saline and HDM-challenged mice (HDM-sensitized, saline-challenged vs. HDM-sensitized, HDM-challenged: 1.6 ± 0.3 platelets/mm

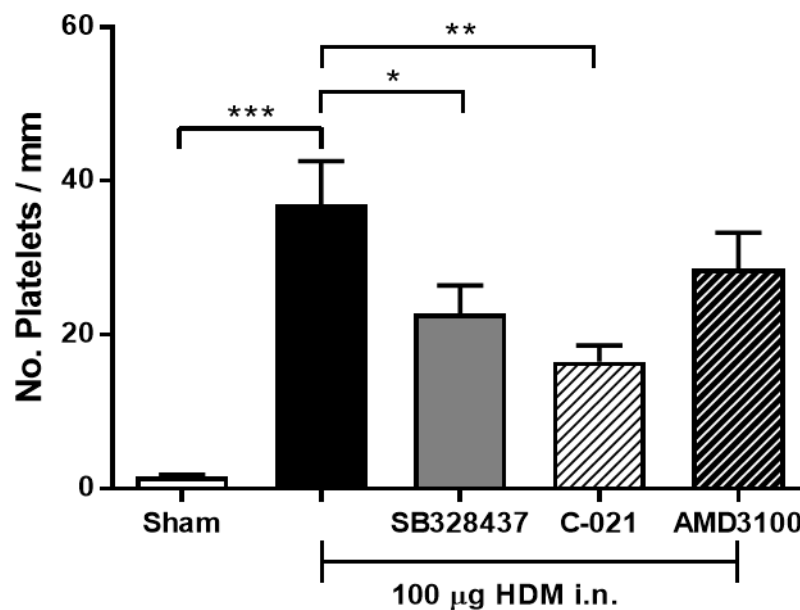


Figure 6.3: The effects of chemokine receptor antagonists on platelet migration after allergen challenge, following a recovery period.

HDM-sensitized mice (25 µg HDM on days 0, 1, 2, 3, 4, 7, 8, 9, 10, and 11) were administered vehicle, SB328437 (30 mg/kg i.p.), C-021 (30 mg/kg i.p.) or AMD3100 (10 mg/kg i.p.) on day 20, 30 minutes before saline or HDM (100 µg i.n.) challenge. On day 21, the lungs were dissected, formalin fixed and paraffin wax embedded. Sections of lung were immunohistochemically stained using platelet specific anti-mouse CD42b antibody. The number of platelets in the airway wall was quantified and expressed per mm along the airway wall. Data presented as mean \pm SEM, $n = 8-12$, * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$.

vs. 37.0 ± 5.7 platelets/mm, $P < 0.001$), thus showing the impact of a longer recovery period on platelet migration to the lungs (Figure 6.3). Administration of the CCR3 receptor antagonist (SB328437) and the CCR4 receptor antagonist (C-021), caused a significant reduction in the number of migrated platelets within the airway walls (vehicle vs. SB328437: 37.0 ± 5.7 platelets/mm vs. 22.8 ± 3.6 platelets/mm, $P < 0.05$ and vehicle vs. C-021: 37.0 ± 5.7 platelets/mm vs. 16.5 ± 2.1 platelets/mm, $P < 0.01$), suggesting that CCR3 and CCR4 receptor activation is important for platelet migration following allergen challenge in the lung.

The CXCR4 receptor antagonist (AMD3100), had no significant effect on the number of platelets within the airway walls (vehicle vs. AMD3100: 37.0 ± 5.7 platelets/mm vs. 28.6 ± 4.7 platelets/mm), suggesting the CXCR4 receptor is not involved in platelet migration following allergen challenge to the lung.

The number of PMNs within the airway walls of mice administered the chemokine receptor antagonists, was quantified to study their effects on granulocyte recruitment following allergen challenge. There was a significant increase in PMN accumulation in HDM-challenged compared to saline-challenged mice that had been previously sensitized to allergen (HDM-sensitized, saline-challenged vs. HDM-sensitized, HDM-challenged: 10.1 ± 2.9 PMN/mm vs. 47.3 ± 8.3 PMN/mm, $P < 0.001$) (Figure 6.4). Neither SB328437, nor C-021 had an effect on PMN cell recruitment, suggesting that platelet recruitment following allergen challenge, via CCR3 and CCR4 receptors, is independent of PMN cell recruitment. The administration of the CXCR4 receptor antagonist (AMD3100) significantly reduced PMN cell accumulation to the airway walls following allergen challenge (HDM challenged + vehicle vs. HDM challenged + AMD3100: 47.3 ± 8.3 PMN/mm vs. 25.1 ± 3.2 PMN/mm, $P < 0.05$), suggesting PMN migration into tissue occurs independently of platelet migration via the CXCR4 receptor.

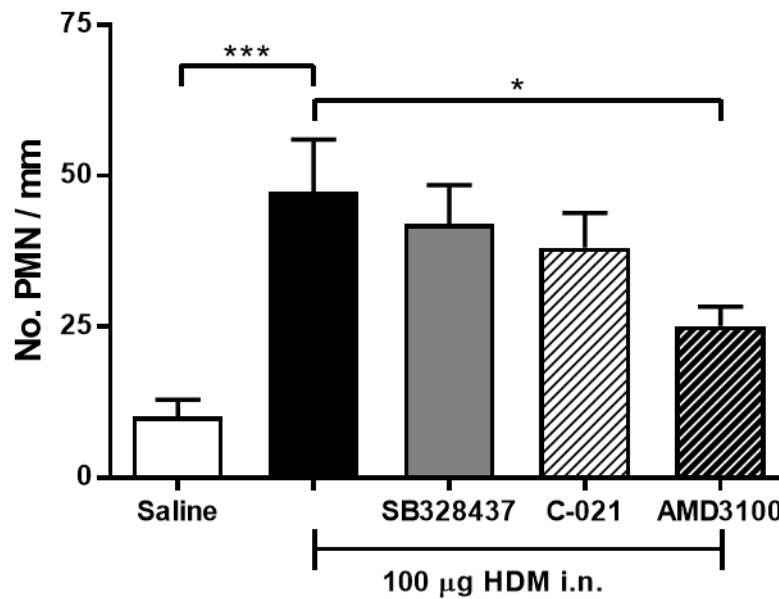


Figure 6.4: The effects of chemokine receptor antagonists on PMN migration after allergen challenge.

HDM-sensitized mice (25 µg HDM on days 0, 1, 2, 3, 4, 7, 8, 9, 10, and 11) were administered vehicle, SB328437 (30 mg/kg i.p.), C-021 (30 mg/kg i.p.) or AMD3100 (10 mg/kg i.p.) on day 20, 30 minutes before saline or HDM (100 µg i.n.) challenge. On day 21, the lungs were dissected, formalin fixed and paraffin wax embedded. Sections of lung were immunohistochemically stained for platelets and counter stained with haematoxylin. The number of PMNs in the airway wall was quantified and expressed per mm along the airway wall. Data presented as mean \pm SEM, $n = 8$, * = $P < 0.05$ and *** = $P < 0.001$.

6.1.3 Bleeding times after chemokine receptor antagonist treatment

In order to assess if the administration of chemokine receptor antagonists affected the critical physiological role of platelets in haemostasis, bleeding times of mice were measured. An increase in bleeding time would indicate that platelet aggregation is affected by chemokine receptor antagonist treatment. Following HDM-sensitization and a one week recovery period, mice were treated with vehicle (sham group), SB328437 (30 mg/kg), C-021 (30mg/kg) or AMD3100 (10mg/kg) and challenged with HDM (100 µg i.n.). 24 hours later, mice were anaesthetized, the tail was resected (2mm from the distal end) and the tail immediately immersed in saline (Greene et al., 2010). The time taken for cessation of bleeding was recorded.

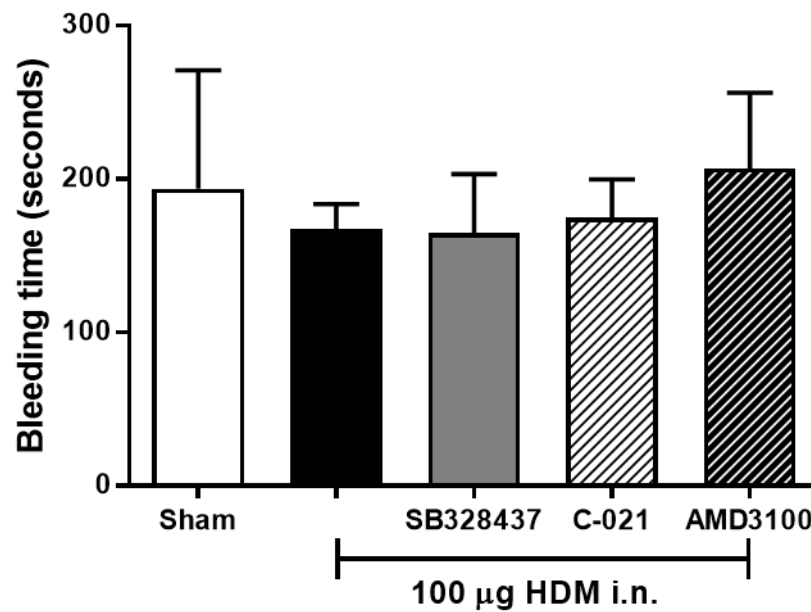


Figure 6.5: The effects of chemokine receptor antagonists on bleeding times following allergen challenge after a recovery period.

HDM-sensitized mice (25 µg HDM on days 0, 1, 2, 3, 4, 7, 8, 9, 10, and 11) were administered vehicle, SB328437 (30 mg/kg i.p.), C-021 (30 mg/kg i.p.) or AMD3100 (10 mg/kg i.p.) on day 20, 30 minutes before saline or HDM (100 µg i.n.) challenge. On day 21, the tail was resected and immersed in warm saline. The time until cessation of bleeding was measured. Data presented as mean ± SEM and n = 8-12.

There were no differences between vehicle and SB328437, C-021 and AMD3100 groups (HDM challenged + vehicle vs. HDM challenged + SB328437 or C-021 or AMD3100: 167.5 ± 16.4 seconds vs. 165.1 ± 38.2 seconds or 175.1 ± 24.2 seconds or 207.0 ± 49.3 seconds) (Figure 6.5), indicating that chemokine receptor antagonists did not interfere with haemostasis.

6.2 The effects of platelet chemokine receptor antagonists on platelet recruitment and accumulation to the cremaster muscle following allergen challenge

6.2.1 The effects of platelet chemokine receptor antagonists on leukocyte recruitment to the cremaster muscle

Using the intravital microscopy model created in section 5.2, the effects of chemokine receptor antagonists on leukocyte recruitment in response to allergen challenge was investigated. HDM-sensitized mice were administered either SB328437 (30 mg/kg), C-021 (30mg/kg) or AMD3100 (10mg/kg) (all i.p.), followed 30 minutes later with the administration of HDM (100 µg s.c.) to the scrotum. After 24 hours the cremaster muscle was dissected and 3 separate post capillary venules were recorded per mouse. In the sham group, mice were sensitized to HDM, before administration of vehicle (i.p.) and saline challenged (s.c.) on the scrotum, with the cremaster muscle dissected on day 14. There was a significant increase in the presence of leukocytes in extravascular compartments of cremaster muscle in HDM challenged than in saline challenged mice (saline challenged vs. HDM challenged: 3.0 ± 0.6 cells/30 µm² vs. 15.3 ± 1.8 cells/30 µm², $P < 0.001$) (Figure 6.6).

The administration of the CCR3 and CCR4 antagonists SB328437 and C-021 had no effect on leukocyte recruitment to the cremaster muscle following allergen challenge (Figure 6.6). The administration of the CXCR4 antagonist (AMD3100) resulted in a significant decrease in leukocyte recruitment to the cremaster muscle (HDM challenged vs. AMD3100: 15.3 ± 1.8 cells/30 µm² vs. 4.3 ± 0.8 cells/30 µm², $P < 0.001$), indicating that the CXCR4 receptor is important for leukocyte recruitment following allergen challenge in this model.

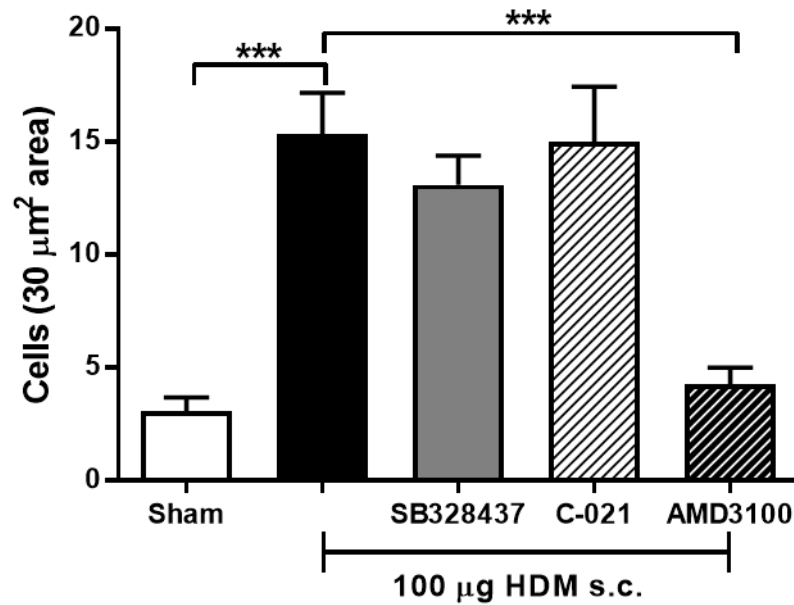


Figure 6.6: The effects of chemokine receptor antagonists on leukocyte accumulation following allergen challenge in the cremaster muscle.

HDM-sensitized mice (25 μg HDM on days 0, 1, 2, 3, 4, 7, 8, 9, 10, and 11) were administered vehicle, SB328437 (30 mg/kg i.p.), C-021 (30 mg/kg i.p.) or AMD3100 (10 mg/kg i.p.) on day 13, 30 minutes before saline or HDM (100 μg) challenge s.c. on the scrotum. On day 14, anti-mouse CD49b PE-conjugated antibody was administered (i.v.), the cremaster muscle was dissected and a minimum of 3 post capillary venules recorded for 10 seconds. The number of leukocytes in extravascular compartments was quantified and expressed per μm^2 , $n = 6-8$ and *** = $P < 0.001$.

6.2.2 The effects of chemokine receptor antagonists on intravascular platelet recruitment and accumulation in the cremaster muscle

Platelet adhesion and platelet rolling events in response to HDM challenge were recorded in HDM-sensitized mice treated with chemokine receptor antagonists. In the sham group, mice were sensitized to HDM, before administration of vehicle (i.p.) and saline challenged (s.c.) on the scrotum, with the cremaster muscle dissected on day 14. Comparing saline challenge and HDM challenge, significantly more platelet adhesion and platelet rolling events were recorded (saline-challenged vs. HDM-challenged: 0.1 ± 0.1 cells/ $30 \mu\text{m}^2$ vs. 1.1 ± 0.1 cells/ $30 \mu\text{m}^2$, platelet adhesion, $P < 0.001$; and 7.8 ± 1.1 rolling/10 seconds vs. 20.7 ± 2.3 rolling/10 seconds, platelet rolling events, $P < 0.001$) (Figure 6.7).

There were significantly fewer platelet adhesion and platelet rolling events in SB328437 treated groups (HDM challenge vs. SB328437: 1.1 ± 0.1 cells/ $30 \mu\text{m}^2$ vs. 0.6 ± 0.2 cells/ $30 \mu\text{m}^2$, platelet adhesion, $P < 0.05$; and 20.7 ± 2.3 rolling/10 seconds vs. 12.3 ± 2.9 rolling/10 seconds, platelet rolling events, $P < 0.05$), indicating that the CCR3 receptor is involved in platelet recruitment to sites of allergic inflammation (Figure 6.7). Administration of the CCR4 receptor antagonist C-021, had no effect on platelet adhesion and platelet rolling events in the cremaster muscle following allergen challenge (Figure 6.7). Administration of AMD3100, a CXCR4 receptor antagonist, also had no effect on platelet adhesion and platelet rolling events (Figure 6.7). Given that the administration of the CXCR4 antagonist caused a decrease in leukocyte recruitment to the cremaster muscle, this might suggest that platelet recruitment to the cremaster muscle is independent of leukocyte recruitment in this model.

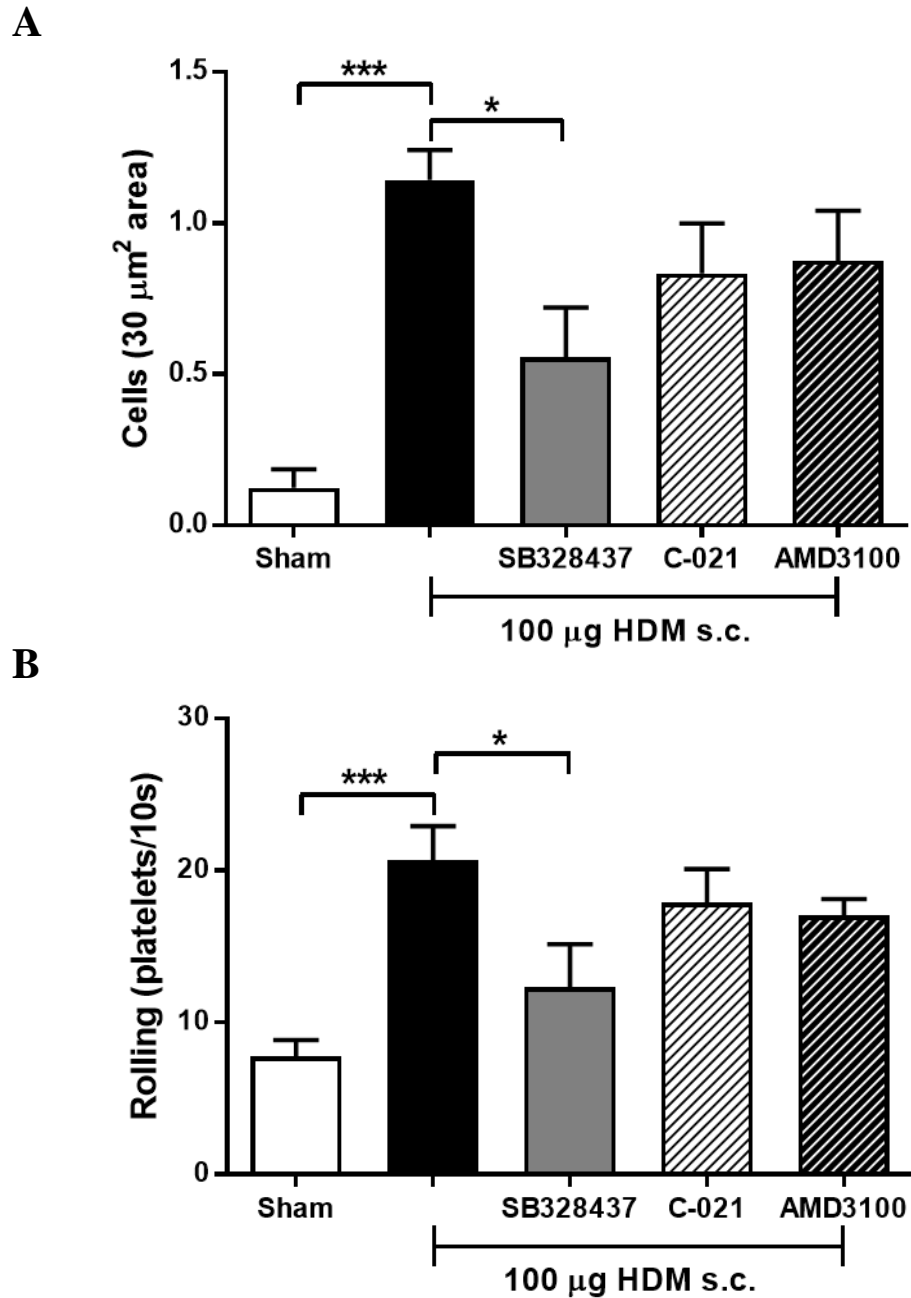


Figure 6.7: The effects of chemokine receptor antagonists on platelet adhesion and platelet rolling events in the cremaster muscle.

HDM-sensitized mice (25 μg HDM on days 0, 1, 2, 3, 4, 7, 8, 9, 10, and 11) were administered vehicle, SB328437 (30 mg/kg i.p.), C-021 (30 mg/kg i.p.) or AMD3100 (10 mg/kg i.p.) on day 13, 30 minutes before saline or HDM (100 μg) challenge s.c. to the scrotum. On day 14, anti-mouse CD49b PE-conjugated antibody was administered (i.v.), the cremaster muscle was dissected and a minimum of 3 post capillary venules recorded for 10 seconds. A- the number of platelet adhesion events was quantified and expressed per μm^2 . B- the number of platelet rolling events during each 10 second video was quantified. Data are presented as mean \pm SEM, $n = 6-8$, * = $P < 0.05$ and *** = $P < 0.001$.

6.2.3 The effects of chemokine receptor antagonists on extravascular platelet accumulation following allergen challenge in the cremaster muscle

The cremaster muscle of chemokine receptor antagonist treated mice, was harvested 24 hours after allergen challenge. The cremaster muscle was then processed for immunohistochemical staining using a platelet specific anti-mouse CD42b antibody. There was a significant increase in extravascular platelets in the cremaster muscle of HDM-sensitized and challenged mice compared to HDM-sensitized saline-challenged mice (saline challenged vs. HDM challenged: 1.6 ± 0.4 platelets/mm² vs. 12.4 ± 1.4 platelets/mm², $P < 0.001$) (Figure 6.8). Treatment with the CCR3 receptor antagonist (SB328437) decreased platelet accumulation within the extravascular compartments of cremaster muscle (HDM

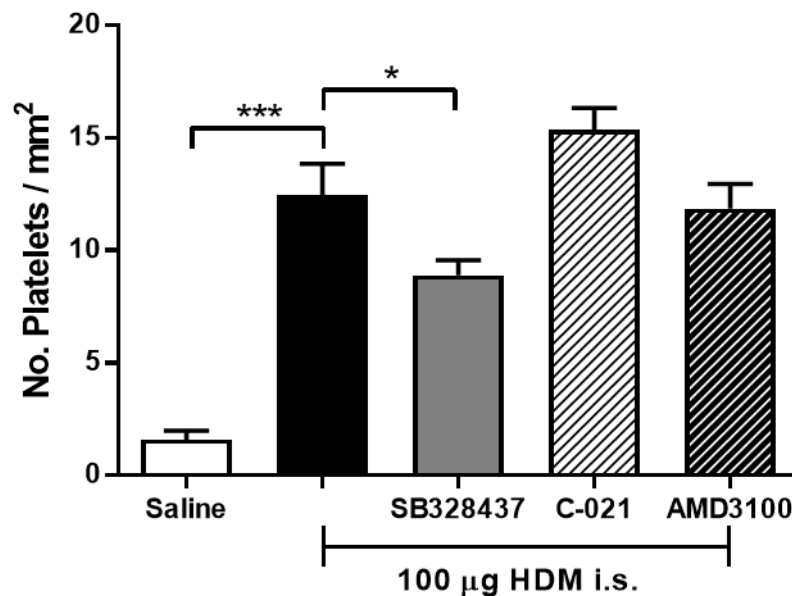


Figure 6.8: The effects of chemokine receptor antagonists on platelet migration following allergen challenge in the cremaster muscle.

HDM-sensitized mice (25 µg HDM on days 0, 1, 2, 3, 4, 7, 8, 9, 10, and 11) were administered vehicle, SB328437 (30 mg/kg i.p.), C-021 (30 mg/kg i.p.) or AMD3100 (10 mg/kg i.p.) on day 13, 30 minutes before saline or HDM (100 µg) challenge s.c. to the scrotum. On day 14, the cremaster muscle was dissected, formalin fixed and paraffin wax embedded. Sections of lung were immunohistochemically stained using platelet specific anti-mouse CD42b antibody. The number of platelets in extravascular compartments of cremaster muscle was quantified and expressed per mm² of tissue. Data are presented as mean ± SEM, $n = 6-8$, * = $P < 0.05$ and *** = $P < 0.001$.

challenged + vehicle vs. HDM challenged + SB328437: 12.4 ± 1.4 platelets/mm² vs. 8.9 ± 0.7 platelets/mm², $P < 0.05$), supporting intravital microscopy findings on platelet adhesion and platelet rolling events.

CCR4 receptor antagonism decreased platelet migration to the lungs of HDM-sensitized mice challenged with HDM (Figure 6.3), although such an effect was absent in the intravital microscopy cremaster muscle model. The administration of the CXCR4 receptor antagonist (AMD3100) again had no effect on the extravascular accumulation of platelets, further supporting the inference that leukocyte recruitment to the cremaster muscle occurs via separate migratory pathways involving chemokine receptors compared to platelets.

6.2.4 *The effects of s.c. eotaxin administration to the scrotum of HDM-sensitized mice*

CCR3 receptor antagonism caused a reduction in platelet rolling, platelet adhesion and platelet accumulation into the cremaster muscle; moreover a similar inhibitory effect was observed on platelet accumulation into the lungs, in mice sensitized and exposed to HDM. Furthermore, platelets harvested from allergen-sensitized mice migrated towards the CCR3 ligand eotaxin to a greater extent than platelets harvested from sham-sensitized mice *in-vitro*, and a doubling in CCR3 expression was also revealed on the surface of allergen-sensitized platelets. The role of the CCR3 receptor was further investigated by administering a CCR3 ligand eotaxin *in-vivo*, and it was hypothesised that CCR3 receptor activation via exogenous eotaxin administration, might elevate platelet recruitment in HDM-sensitized mice compared to sham-sensitized mice. Based on previous literature, following the HDM-sensitization protocol, mice were challenged with eotaxin (0.1 µg, s.c. to the scrotum) and 4 hours later the cremaster muscle was dissected and prepared for intravital microscopy as described elsewhere (Riffo-Vasquez et al., 2012).

The number of leukocytes in extravascular compartments was significantly higher in sham-sensitized mice exposed to eotaxin compared to saline (sham-sensitized mice + vehicle vs. sham-sensitized mice + eotaxin: 0.7 ± 0.1 cells/30 µm² vs. 2.1 ± 0.5 cells/30 µm², $P < 0.05$)

(Figure 6.9A). However, HDM-sensitization had no further effect (Figure 6.9A). Although HDM-sensitization had no effect on platelet adhesion and platelet rolling events, eotaxin administration appeared to cause an increase in intravascular platelet rolling and adhesion (Figure 6.9B and C). Eotaxin administration to mice caused an inflammatory response, which involved both leukocyte and platelet recruitment into the cremaster muscle assessed by intravital microscopy. HDM-sensitization of mice had no effect on the action of eotaxin in this

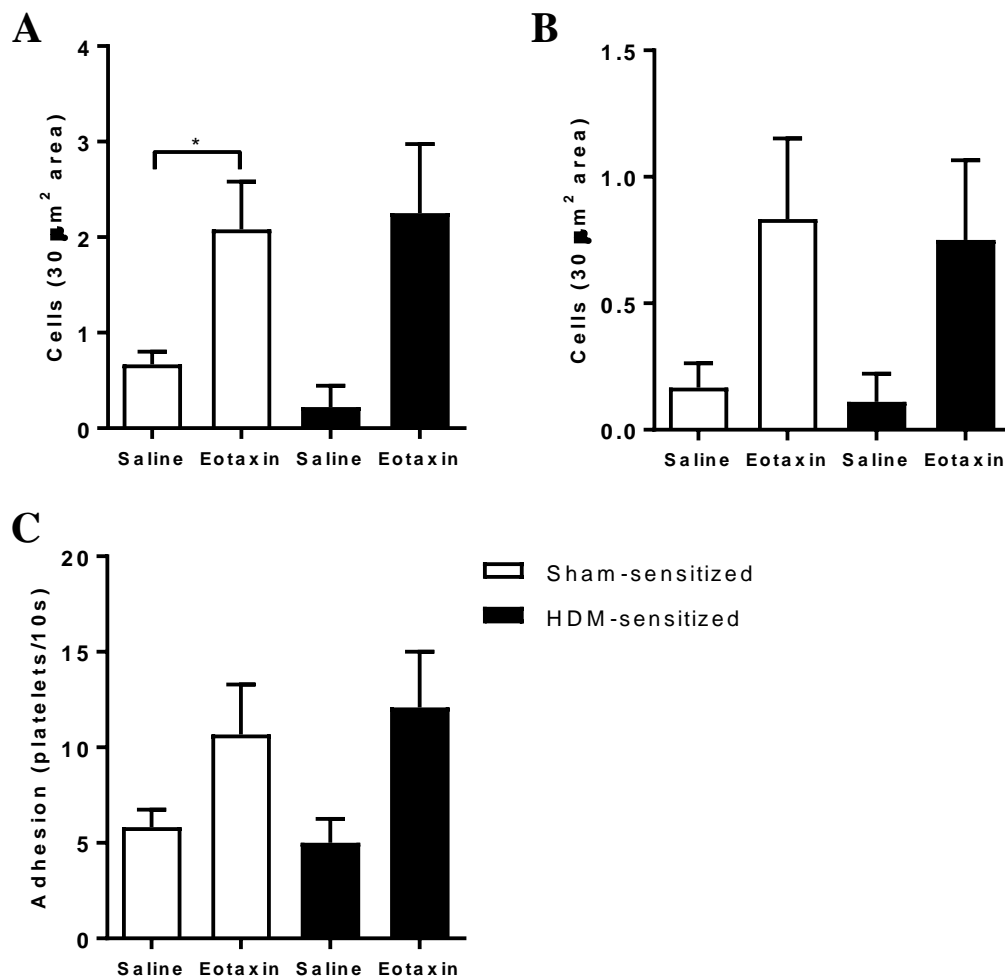


Figure 6.9: The effects of eotaxin on leukocyte recruitment, platelet adhesion and platelet rolling events in the cremaster muscle.

Sham-sensitized and HDM-sensitized mice (25 μg HDM on days 0, 1, 2, 3, 4, 7, 8, 9, 10, and 11) were administered eotaxin (100 ng s.c. to the scrotum) on day 14. 4 hours later the cremaster muscle was dissected and a minimum of 3 post capillary venules recorded for 10 seconds. A- the number of leukocytes in extravascular compartments was quantified and expressed per μm^2 . B- the number of platelet adhesion events was quantified and expressed per μm^2 . C- the number of platelet rolling events during each 10 second video was quantified. Data are presented as mean \pm SEM, $n = 4$ and * = $P < 0.05$.

model, indicating either that HDM-sensitization of mice does not cause increased platelet CCR3 receptor activity, or that the dose of eotaxin delivered was supramaximal, masking any potential synergism that may occur between allergen sensitization and chemokine receptor expression and activity (Sehmi et al., 2003; Francis et al., 2007; Wang et al., 2007). Chemokine receptor antagonist administration to allergen-sensitized mice exposed to allergen, produced effects on platelet and leukocyte recruitment. The administration of the CCR3 receptor antagonist (SB328437) prevented platelet recruitment and migration in response to allergen challenge, suggesting a role for CCR3 in allergen induced platelet responses. Administration of the CCR3 ligand eotaxin *in-vivo*, caused an inflammatory response involving both platelets and leukocytes. CCR4 receptor antagonist (C-021) administration reduced platelet migration following allergen challenge to the lung, although this effect was not observed in response to allergen challenge in the cremaster muscle, which may suggest that a different allergic inflammatory pathway governs platelet responses in different organs. The administration of the CXCR4 receptor antagonist (AMD3100) had no effect on platelet responses in allergic inflammatory models. However, AMD3100 caused a reduction in leukocyte migration following allergen challenge which occurs independently of platelet migration via the CXCR4 receptor. Platelet chemokine receptor expression, *in-vitro* functional activity and *in-vivo* activity in allergic inflammatory models was demonstrated, suggesting that chemokine receptors may be involved in the platelet migration that occurs in a number of inflammatory conditions (Metzger et al., 1987; Jeffery et al., 1989; Boilard et al., 2010; Langer et al., 2012).

Chapter 7 Discussion

7.1 Platelet recruitment in fatal asthmatic patients

Platelets have well established roles in thrombosis and haemostasis, which has led to the successful development of anti-platelet and anti-thrombotic drugs for a number of conditions (Eikelboom et al., 2012). Most notably the inhibition of platelet aggregation via the platelet P2Y₁₂ receptor antagonists such as clopidogrel and prasugrel, are established standard therapies for patients with acute coronary syndromes and those undergoing percutaneous coronary intervention. Low dose aspirin inhibits cyclooxygenase-1 production of TXA₂ but the inhibition of cyclooxygenase-2 production of prostacyclin (PGI₂) by endothelial cells is recovered through *de novo* synthesis of cyclooxygenase-2, and the altered ratio of PGI₂/TXA₂ leads to reduced platelet aggregation and vasoconstriction. Aspirin can be given prophylactically to patients at high risk of cardiovascular disease and stroke, which reduces the chance of embolus formation. The pharmacological control of platelet aggregatory responses has led to improved clinical outcomes in patients suffering with disease, therefore novel drug treatments to combat platelet inflammatory responses, may produce therapeutic effects that do not affect haemostasis.

In this thesis, human lung samples from patients with asthma were stained and quantified for the presence of platelets. Airway provocation (of unreported stimuli) in patients who died of asthma caused increased platelet recruitment to the lung parenchyma (alveoli), compared to tissue sections from non-fatal asthmatic and non-atopic donors. These platelets did not appear within blood vessels, but rather in abluminal regions of the alveolar wall, and indeed within alveolar air spaces. Furthermore, no platelet aggregates were observed in the human lung, suggesting that platelet accumulation was not a result of an aggregatory response, but rather an inflammatory response. Whilst there are limits with two-dimensional histological analysis to fully conclude the positioning of platelets within the lung structure, such a recruitment of platelets has been observed in the lungs of allergen-sensitized mice after allergen exposure, which was IgE FcεR1-dependant (Pitchford et al., 2008). Clinically, platelets have been reported in extravascular compartments of bronchial biopsy samples,

supporting the deduction that platelet recruitment to the lungs occurs in allergic states (Jeffery et al., 1989). However, this is the first study in which the presence of platelets has been quantified via antigen-specific immunohistochemistry, comparing different patient cohorts. It is interesting to note that such a phenomenon has been observed in other disease states, where platelets were found to have migrated extravascularly into inflamed tissue. Inhalation of LPS and bacteria can promote migration of platelets into airspaces; whilst other studies reveal the presence of platelets in the synovial fluid of patients with rheumatoid arthritis and the brain of patients with multiple sclerosis (Boilard et al., 2010; Langer et al., 2012; Ortiz-Muñoz et al., 2014; Amison et al., 2018b).

Circulating platelets have diminished stores of mediators and a heightened responsiveness in allergic states, which is thought to lead to the migration of platelets to the lungs of asthmatic patients (Taytard et al., 1986; Page and Pitchford, 2014; Amison et al., 2015). Indeed platelets are present in bronchoalveolar lavage fluid of patients with asthma, therefore it was hypothesised that non-fatal asthmatic patients would have increased platelet staining events in the lungs compared to healthy patients (Metzger et al., 1987). A moderate increase in platelet recruitment was observed, however this effect was not significant. Carroll and colleagues measured eosinophil staining events around membranous small airways and determined no significant differences between healthy and non-fatal asthmatic patients, and a significant increase in the number of eosinophils in fatal asthmatic patients compared to healthy donors (Carroll et al., 1997). Although the recruitment of platelets was not increased in non-fatal asthmatic patients compared to healthy patients, this recruitment mirrors eosinophils recruitment in healthy, non-fatal and fatal asthmatic patients. Such studies suggest the difficulties in measuring cellular recruitment to the lungs that occurs in the absence of a temporally relevant stimulus. For example, platelet activation, and peripheral thrombocytopenia occur in patients with asthma during allergen challenge with the house dust mite allergen Der p 1, presumably as a result of localized pulmonary platelet recruitment (Kowal et al., 2006). Further studies would therefore be required to obtain lung biopsies from

patients with asthma having undergone antigen exposure in clinically controlled conditions to fully elucidate the nature of platelet recruitment and migration into lung tissue.

Sections of human lung did not have sufficient amounts of airway wall to confidently assess platelet recruitment to this lung structure, however single non-aggregated platelets were observed within the airway walls of non-fatal and fatal asthmatic patients. Platelets are important for leukocyte recruitment (Lellouch-Tubiana et al., 1988; Pitchford et al., 2003, 2005; Dürk et al., 2013; Tian et al., 2015; Takeda et al., 2016; Cardenas et al., 2018), bronchospasm (Halonen et al., 1981; Coyle et al., 1990; Keir et al., 2015; Cardenas et al., 2018) and airway wall remodelling (Pitchford et al., 2004) in animal models of allergic inflammation, demonstrating their potential roles in disease progression. Understanding the mechanisms that underlie this migration of platelets to sites of allergic inflammation could therefore present novel targets for allergic inflammatory diseases because platelets might directly contribute to the pathophysiology of asthma.

7.2 *In-vitro* and *in-vivo* model development

7.2.1 *In-vitro* platelet chemotaxis assays

To begin investigating the migration of platelets to allergic inflammatory sites, an existing *in-vitro* model of platelet chemotaxis was used to test the effects of different stimuli on platelet chemotaxis. The microplate chemotaxis assay (Neuroprobe) is a high throughput model, where cells and chemokine are separated by a porous filter. The model has previously been used to show human platelet chemotaxis towards fMLP and similar use of Boyden chambers reveal platelet chemotaxis toward OVA, when platelets are harvested from OVA-sensitized mice, or to specific allergen to which human donors with asthma are allergic (Czapiga et al., 2005; Pitchford et al., 2008). However, this assay lacks flexibility required for assay development, which is confined to comparing pore size, incubation times and cell number. In comparison, the use of transwell chambers and inserts provides the experimenter with a degree of control to adjust the volumes of solution within each compartment, which might influence fluid contact across the membrane due to pressure differences, and therefore modulate chemokine dispersion from the bottom compartment. Increasing the volume in the bottom well increased the sensitivity of the assay (conducted in house), and the assay was further optimized by adjusting the 90 minute incubation time, which was originally determined using the Neuroprobe microplate system, to a 45 minute incubation time for platelets. The transwell chemotaxis assay successfully showed human platelet chemotaxis towards a number of stimuli, notably fMLP, where a characteristic bell-shaped concentration response was observed. Furthermore, the introduction of a chemokinetic control in the bottom well, inhibited directional movement. Human platelet chemotaxis towards eotaxin (CCR3 ligand), MDC (CCR4 ligand), and SDF-1 α (CXCR4 ligand) were all demonstrated. This provided further rationale to also investigate the effects of these chemokine receptors in *in-vivo* models. However, this model also has its limitations. The release of chemokine *in-vivo* forms a steady concentration gradient within a tissue, whereas with the microplate and transwell chemotaxis assays, initially two compartments with 0% chemokine and 100%

chemokine are separated by an artificial surface producing a steep gradient, which likely shallows over the incubation period. Furthermore, a proportion of platelets may ‘fall through’ the porous membrane by means of insufficient platelet adhesion to substrate and gravity.

In order to further elucidate platelet chemotaxis *in-vitro*, the microfluidic chemotaxis assay (Ibidi) was considered as it allows single cell tracking, which allows several measurements of motility to be attained that cannot be produced with the microplate or transwell chemotaxis assays. In the microfluidic chemotaxis assay a gradual horizontal concentration gradient can be generated, eliminating gravitational effects and perhaps more closely mimicking chemokine gradients produced *in-vivo*. Kraemer and colleagues studied platelet chemotaxis towards CXCR4 in an assay that shares some similarities. They successfully measured platelet chemotaxis on fibrinogen-coated chamber slides to an agarose pellet containing SDF-1 α (Kraemer et al., 2010). Platelet chemotaxis was measured over a 3 hour period, which in most cases platelets would migrate less than 5 μm in total. They also allowed platelets to settle for 30 minutes, then wash through to remove non-adherent platelets, however the platelets that are non-adherent may be expressing a different phenotype to those that have adhered (Kraemer et al., 2010). The aim was therefore to produce a similar assay that could detect motile platelets and assess several facets of platelet chemotaxis.

After applying platelets to the microfluidic well, one of the main challenges was to cause sufficient platelet adherence to the surface of the well. A lack of adherence to the slide resulted in platelets moving freely in solution, which would move less directionally and were difficult to focus. It was also important to prevent platelets adhering too firmly to the surface of the well, because too firm an adhesion would prevent the movement of platelets and likely lead to platelet activation. Allowing the platelets to settle on the surface of the well increased the number of sufficiently adhered platelets, although a proportion of platelets still remained non-adhered. Initial studies using a mouse serum coating of microfluidic wells successfully enabled platelet chemotaxis to be measured. However, when using the same mouse serum solution at a different site, platelets tended to adhere too firmly to the surface of the well.

Clearly, unknown differences in laboratory conditions affected the ability to transfer the assay. Consequently, different well coatings were investigated with a more uniform composition to reduce the chances of variability. Fibrinogen coating of microfluidic slides produced the greatest platelet chemotaxis effect compared to other coating solutions, and was consequently used for further studies. Indeed resting platelets can bind to fibrinogen independently of platelet activation, and therefore has been used previously for platelet chemotaxis investigations (Savage and Ruggeri, 1991; Zaidi et al., 1996; Kraemer et al., 2010).

Very recently, platelets have been reported to migrate along the luminal surface of blood vessels, to act as mechano-scavengers that collect and bundle bacteria to influence neutrophil behaviour (Gaertner et al., 2017). This same group developed a similar methodology for measuring platelet shape change and chemotaxis *in-vitro* on immobilized blood plasma components. The balanced co-absorption of pro-adhesive fibrinogen and anti-adhesive albumin (Park et al., 1991), presumably provides a mixture to allow cellular polarization and integrin dependent adhesion of spread platelets (Gaertner et al., 2017). Conditions where adhesion dominates over traction have historically been used to measure platelet spreading and adhesion, and thus migration is likely impeded, and have been reported for other cell types (Palecek et al., 1997; Gupton and Waterman-Storer, 2006). Future experiments using the Ibidi chambers would involve the use of fibrinogen with varying concentrations of albumin, to elucidate optimum conditions that can be repeatedly recreated and not dependent on batches of mouse serum.

When using the microfluidic chemotaxis assay, several different measurements are generated that can be used as a measure of platelet chemotaxis, which require careful consideration before choosing as they exhibit different elements of platelet motility. Interestingly, when platelets migrated in the presence of a chemokine, the centre of mass, Euclidean distance and directionality of migrating platelets all increased simultaneously, indicating a relationship between these parameters. Both the accumulated distance and velocity of migrating platelets also appear to share a relationship (as expected). For validation

studies, the centre of mass Y coordinate was selected as a general measure of platelet chemotaxis, as it specifically shows movement in the direction of chemokine.

Platelets tended to migrate toward the end of the well that buffer or chemokine was added. This phenomenon may be attributed to circulating flow caused by the pipetting motion of adding to the well. The assay was originally validated for leukocytes (Johnsson et al., 2014), which are larger cells ($\sim 10\ \mu\text{m}$) that are often more firmly adhered to the surface and therefore less easily effected by flow. Platelets however are smaller ($\sim 3\ \mu\text{m}$) and less firmly adhered, so are more likely effected by flow within a well with a total height of $400\ \mu\text{m}$. Minimizing the flow or increasing the platelet chemotactic effect, will improve the window between control and chemokine stimuli, which is key in validating the microfluidic chemotaxis assay. Nonetheless this assay enabled platelets to migrate $40\ \mu\text{m}$ over a period of 8 minutes towards chemokine (Figure 4.15). This is the first time the microfluidic chemotaxis assay has been employed for measuring platelet chemotaxis, thus the model requires considerable validating experiments before in depth studies can be performed, such as investigating the effects of allergen sensitization on platelet chemotaxis.

7.2.2 Intravital microscopy of mouse cremaster muscle

The observation of platelet chemotaxis measured *in-vitro*, which was elevated in sensitized mice towards eotaxin (discussed below), justified further investigations into platelet motility and migration to sites of allergic inflammation in a more physiologically relevant *in-vivo* system. Intravital microscopy of airways was considered as a possible means to investigate platelet recruitment. Generating clear visualisations of cell recruitment to the lung is possible through the use of sophisticated technologies and adaptations of the lung, however this can be technically challenging due to the vital physiological role of the lungs and their enclosed position within the body (Looney et al., 2011; Looney and Bhattacharya, 2014; Rodriguez-Tirado et al., 2016). Platelets are especially challenging to view due to their small size, and the breathing motion of the lung would greatly impact visualisations of platelet recruitment. Therefore the cremaster muscle was investigated as a means to study platelet

migration as it is easily accessible, motionally stable, and due its thinness, clear visualisations of the vasculature can be obtained. Admittedly, the cremaster muscle and lung architecture and functions are very different, and the testes do not normally become sensitized to allergen, which needs to be considered when interpreting data.

Due to experimental issues when sensitizing mice to OVA that have been explained in the results section, HDM-sensitization was considered to produce an allergic inflammatory response in the cremaster muscle, because this is a clinically relevant source of antigen (Der p 1), which has been used successfully to develop *in-vivo* models of allergen sensitization and inflammation via intranasal inhalation (Gregory et al., 2009; Gregory and Lloyd, 2011). HDM-sensitization caused increased eosinophil recruitment to the lungs, demonstrating that an allergic inflammatory response was generated. However, it was necessary to test whether the sensitization was systemic in nature, given the intended application for intravital microscopy on the cremaster muscle. The successful sensitization of mice was therefore supported by allergic skin tests on the flanks of mice demonstrating that systemic sensitization was achieved. Thus, the HDM sensitization protocol was used for intravital microscopy studies of the mouse cremaster muscle. Following HDM administration s.c. on the scrotum, there was increased leukocyte recruitment and eosinophil migration in HDM-sensitized mice compared to saline-sensitized mice, indicating an allergic inflammatory response was generated in the cremaster muscle.

In saline-sensitized mice challenged with HDM, there were elevated numbers of leukocytes in the cremaster muscle and increased platelet events, when compared to saline-sensitized mice challenged with saline. This indicates that HDM extract alone causes an inflammatory response without prior sensitization, which has been suggested to occur through HDM protease and endotoxin activity (Gregory and Lloyd, 2011). Der p 1 cleaves epithelial tight junctions, which impairs epithelial barrier function and allows delivery of allergen to APCs. HDM extracts also contain endotoxin and Gram-negative *Bartonella* species that are thought to be a source of LPS, which can trigger proinflammatory signals (Gregory and Lloyd,

2011). These components of HDM preparations elucidate the inflammatory responses generated without prior sensitization. Importantly however, when mice were HDM-sensitized and HDM-challenged, both leukocyte and platelet responses were further elevated, suggesting an allergic inflammatory response was generated.

For the first time, platelet recruitment following allergen exposure was measured by intravital microscopy using a platelet specific fluorescent antibody (anti-CD49b-FITC antibody). Because this model offers a motionally stable tissue bed, allowing clear visualisations of platelets in the vasculature, distinct single platelet rolling and platelet adhesion events were recorded following allergen challenge. These events were independent of leukocyte rolling and adhesion as could be determined in merged fluorescent and bright field displays of cremaster muscle. The rolling and adhesion of platelets did not lead to platelet aggregate formation, suggesting that the mechanisms involved in platelet recruitment following allergen challenge are separate from platelet aggregatory responses (Page, 1988).

Single platelets were observed in extravascular compartments of cremaster muscle and in extravascular compartments surrounding airway wall structures, following allergen exposure in HDM-sensitized mice, demonstrating that platelets have the ability to migrate to sites of allergic inflammation, supporting previous studies which have shown platelets localised to inflamed tissue (Metzger et al., 1987; Jeffery et al., 1989; Boilard et al., 2010; Langer et al., 2012). It is noted however, that the physical act of migration itself, through the endothelium was not possible to record here, and the assumption of platelet migration through tissue is based on events preceding diapedesis (e.g. vascular rolling and adhesion) and the post-adhesive quantification of platelet accumulation within the tissue. This is the first report of platelet adhesion in response to exposure to a clinically relevant allergen, which suggests that following allergen challenge, platelets are recruited in a similar manner to leukocytes, leading to increased rolling along venules located in the inflamed area, followed by firm adhesion of platelets to the endothelium and finally diapedesis into extravascular compartments of inflamed tissue. Studies could be taken further by making use of *in-vitro*

endothelial monolayer preparations and flow based systems to record the ability of platelets to undergo diapedesis to physiologically relevant, and allergic stimuli. It is interesting to note however, that platelets might undergo transcellular diapedesis, as revealed in electron microscopy studies of platelets undergoing diapedesis in response to fMLP induced acute inflammation in guinea pigs (Feng et al., 1998).

Platelets are highly specialised for aggregatory responses, although they possess the necessary components to function as immune cells. Platelets appear to contribute to allergic inflammatory responses by propagating the effects of other inflammatory cells. Platelet-dependent leukocyte recruitment to the lung presents a specialised role for platelets in allergic inflammatory responses. Further investigations into the roles of platelets in allergic conditions may uncover additional unique functionalities of platelets that are directly related to platelet function, rather than platelet cooperation of leukocyte dependent pathogenesis. Data from this thesis supports the notion that platelets indeed migrate to sites of allergic inflammation, which may occur through a number of different mechanisms, although the platelet chemokine receptors have been investigated in this instance.

7.3 Platelet chemokine receptors

The overarching aim of this work was to investigate if chemokine receptors expressed on platelets are involved in platelet migration to sites of allergic inflammation. Allergen sensitization induces platelet recruitment and accumulation in allergen-sensitized mice exposed to allergen, via an Fc ϵ RI-dependent process (Pitchford et al., 2008), and it has been suggested that Fc ϵ RI can interact directly with GPCRs, on mast cells (Kuehn and Gilfillan, 2007). In particular MIP-1 α binding to CCR1 has been shown to synergise with Fc ϵ RI in a rat basophilic leukaemia cell line (RBL-2H3 cells), and murine bone marrow derived mast cells (Laffargue et al., 2002; Toda et al., 2004). Furthermore, CCR3 receptor expression in response to allergen on leukocytes has been widely reported (Sehmi et al., 2003; Francis et al., 2007; Wang et al., 2007). Therefore, the influence of allergen sensitization on chemokine receptor expression was initially explored, before functional experiments using novel *in-vitro* and *in-vivo* assays was conducted. Investigations were restricted to chemokine receptors known to be expressed on human platelets: CCR1, CCR3, CCR4, and CXCR4 (Clemetson et al., 2000; Kowalska et al., 2000; Abi-Younes et al., 2001). The roles of each platelet chemokine receptor will be discussed in the following section.

7.3.1 Platelet CCR1 receptor

Stimulation of platelets with MIP-1 α causes increased intracellular calcium concentrations, indicating that the CCR1 receptor is functional on platelets, but has not been explored for motility or shape change (Clemetson et al., 2000). MIP-1 α has been detected in the lungs of asthmatic patients and in the serum of patients with atopic dermatitis, suggesting that the CCR1 receptor may be involved in allergic inflammatory responses (Alam et al., 1996; Tillie-Leblond et al., 2000; Kaburagi et al., 2001). MIP-1 α has been shown to induce chemotaxis of other cell types, therefore the ability of MIP-1 α to induce platelet chemotaxis via the CCR1 receptor was investigated (Phillips et al., 2003; Toda et al., 2004).

Murine platelet CCR1 receptor expression was demonstrated via flow cytometry and western blot analysis. Sensitization of mice to allergen appeared to have no effect on CCR1 receptor expression, indicating neither surface expression of CCR1 or total cell CCR1 content is effected by sensitization. Platelet chemotaxis via CCR1 receptor activation was investigated using the microplate chemotaxis assay. MIP-1 α was unable to induce platelet chemotaxis in this assay. Furthermore neither the sensitization of mice to OVA, nor subsequent exposure of OVA-sensitized mice with OVA induced CCR1 receptor dependent platelet chemotaxis (via MIP-1 α stimulation). Due to the lack of effect on platelet CCR1 receptor expression and chemotaxis, the CCR1 receptor was not investigated further.

It has been suggested that co-stimulation of CCR1 and CCR3 receptors impacts eosinophil recruitment to the lungs of asthmatic patients, so although CCR1 receptor stimulation alone did not induce platelet chemotaxis, it is possible that activating CCR1 along with CCR3 receptor on platelets, may present a function of CCR1 on platelet chemotaxis, which was not tested in this thesis (Sabroe et al., 2000; Phillips et al., 2003).

7.3.2 Platelet CCR4 receptor

Stimulation of platelets with MDC causes platelet activation as measured by calcium signalling, granule content release, platelet shape change and low level platelet aggregation, demonstrating the functional activity of the CCR4 receptor on platelets (Clemetson et al., 2000; Abi-Younes et al., 2001; Gear et al., 2001). Activation of the CCR4 receptor can induce chemotaxis in other cell types, therefore the ability of the CCR4 receptor to induce platelet chemotaxis was investigated (Colantonio et al., 2002; Juremalm et al., 2002; Kim et al., 2002).

Mouse platelet CCR4 receptor surface expression and total cell CCR4 content was confirmed through flow cytometry and western blot analysis, respectively. Mouse and human platelet chemotaxis was measured to MDC in each of the chemotaxis assays tested, demonstrating the ability of CCR4 activation to induce platelet chemotaxis. Patients with asthma have increased CCR4 receptor expression on T cells (Vijayanand et al., 2010), which

suggests allergen sensitization causes increased CCR4 receptor function in allergic states. However, the sensitization of mice had no effect on the expression of CCR4 on platelets or the modulation of platelet chemotaxis towards MDC. Similarly, the CCR4 receptor antagonist C-021 had no effect on platelet rolling, adhesion or migration to the cremaster muscle following HDM-challenge in HDM-sensitized mice. Conversely, C-021 caused decreased platelet migration to the lungs following allergen challenge of sensitized mice.

Investigations into the role of the CCR4 receptor in allergic inflammatory models has exhibited contrasting effects. Adoptively transferred antigen specific Th2 cells from CCR4 deficient mice, prevented trafficking following allergen challenge in an OVA model of allergic pulmonary inflammation (Mikhak et al., 2009). Similarly, antibody neutralisation of MDC and TARC caused reduced leukocyte recruitment to the lung following allergen challenge (Lloyd et al., 2000; Kawasaki et al., 2001). Furthermore, in mice administered with peripheral blood mononuclear cells from asthmatic patients, CCR4 blocking antibodies caused decreased airway eosinophilia, goblet cell hyperplasia and bronchial hyperreactivity (Perros et al., 2009). In contrast, using a CCR4 receptor antagonist in a guinea pig model of allergic airway inflammation and in an allergic inflammatory model using CCR4 knockout mice, there was no protection against airway inflammation (Chvatchko et al., 2000; Conroy et al., 2003). The differences in CCR4 receptor action in models of allergic inflammation have been suggested to be species dependant (Solari and Pease, 2015). These contrasting effects may reflect the differences in platelet migration to the cremaster muscle and the lung following allergen challenge.

The CCR4 receptor is believed to have roles in T lymphocyte (Th2 subsets) recruitment to allergic lungs, as MDC is upregulated in lungs following allergen challenge and T cells co-localize with MDC in bronchial biopsy samples from asthmatic patients (Panina-Bordignon et al., 2001; Pilette et al., 2004). Allergic inflammatory responses in the lung are strongly driven by the Th2 immunophenotype, but it is unknown how the HDM-induced allergic inflammatory response generated in the cremaster muscle may compare (Busse and

Rosenwasser, 2003). C-021 administration may impair the Th2 response which could have a downstream effect on platelet migration into the lung. If the inflammatory response generated in the cremaster muscle is not as strongly driven by Th2 T cells, this may explain the lack of effect C-021 caused on platelet migration to the cremaster muscle. However, this theory does not explain why leukocyte recruitment to the lungs and cremaster muscle was unaffected by C-021 treatment. Further investigations into the dose and timing of C-021 administration to sensitized mice, or the use of transgenic mice, may reveal different responses of platelets and leukocytes following allergen challenge via CCR4, which is not appreciated in an acute pharmacological study.

To summarise, the platelet CCR4 receptor is capable of causing platelet chemotaxis, however the role of CCR4 in platelet migration to sites of allergic inflammation requires further elucidation.

7.3.3 Platelet CXCR4 receptor

Treating human platelets with SDF-1 α causes increased intracellular calcium concentrations, P-selectin expression, platelet shape change and low level platelet aggregation, via CXCR4 receptor activation (Clemetson et al., 2000; Kowalska et al., 2000; Gear et al., 2001). Stimulating washed platelets with SDF-1 α has also been shown to induce platelet chemotaxis *in-vitro*, however the role of platelet CXCR4 in allergic inflammatory responses has not been investigated. In murine models of allergic inflammation, treatment of mice with a CXCR4 receptor antagonist or neutralising antibodies, caused decreased airway hyperresponsiveness, eosinophilia and Th2-associated cytokine release (Gonzalo et al., 2000; Lukacs et al., 2002). Indeed in patients presenting with lung eosinophilia, there was increased expression of CXCR4 on eosinophils, suggesting a role of CXCR4 receptor in allergic inflammatory responses (Nagase et al., 2000).

Surface expression and total cell content of CXCR4 was shown on murine platelets, yet sensitization of mice to OVA and HDM had no effect on CXCR4 receptor expression. In the

microplate chemotaxis assay, SDF-1 α did not induce chemotaxis of platelets from sham-sensitized or OVA-sensitized mice, however following allergen challenge there was a subtle increase in platelet chemotaxis to SDF-1 α . Human platelet chemotaxis was also recorded in the transwell chemotaxis assay, demonstrating the ability of SDF-1 α to induce platelet chemotaxis via CXCR4 receptor activation.

The CXCR4 receptor antagonist (AMD3100) had no effect on platelet recruitment and migration in the lung and cremaster muscle following allergen challenge, suggesting that the platelet CXCR4 receptor is not involved in platelet migration to sites of allergic inflammation. AMD3100 administration did however significantly decrease leukocyte recruitment to the cremaster muscle and PMN cell recruitment to the lungs following allergen challenge, indicating CXCR4 receptor has important roles in allergic inflammation. Interestingly, reduced leukocyte recruitment did not result in reduced platelet recruitment and migration to the lungs or cremaster muscle following allergen challenge, indicating platelets can migrate independently of CXCR4 mediated leukocyte migration. It is therefore interesting that the migratory response to allergen *in-vivo* has been reported to commence before initial leukocyte recruitment and migration, whilst at later time points, around 50% of platelets quantified were not complexed to leukocytes, furthermore, there are instances where platelet migration occurred in the absence of leukocytes (Pitchford et al., 2008; Page and Pitchford, 2014). In summary, the platelet CXCR4 receptor is functionally activate, however our data suggests it is not important in platelet migration to sites of allergic inflammation.

7.3.4 Platelet CCR3 receptor

Platelet CCR3 receptor stimulation causes increased intracellular calcium concentrations, low level platelet aggregation and granule release, demonstrating the receptors functional activity on platelets (Clemetson et al., 2000). The CCR3 receptor has well documented roles in allergic inflammation where the receptor is important for eosinophil recruitment to the lungs (Humbles et al., 2002; Fulkerson et al., 2006). Indeed, a number of CCR3 receptor antagonists have been tested clinically on patients with asthma, producing

mixed results (Neighbour et al., 2014; Gauvreau et al., 2018). However, the ability of CCR3 receptor activation to induce platelet chemotaxis and the role of CCR3 on platelet migration to sites of allergic inflammation has not been studied.

Western blot analysis showed mouse platelet CCR3 receptor expression, with no differences between sham-sensitized and HDM-sensitized mice. Similarly, there was no significant difference between sham-sensitized and OVA-sensitized mice platelet CCR3 receptor surface expression as measured by flow cytometry, however there was a doubling in expression following OVA-sensitization. Similarly, CCR3 receptor expression is elevated on other cells in allergic states. Bone marrow CD34⁺ cell expression of CCR3 measured pre and post antigen challenge on dual responder asthmatics, showed increased CCR3 receptor expression (Sehmi et al., 2003). Likewise, CCR3 receptor expression on CD4⁺ T cells was elevated in symptomatic atopic grass pollen-sensitive subjects compared with patients on high-dose allergen injection immunotherapy and healthy controls (Francis et al., 2007). Therefore, increased platelet CCR3 receptor expression following allergen sensitization, may be important in CCR3 mediated platelet migration to allergic inflammatory sites.

CCR3 receptor stimulation via eotaxin caused human and mouse platelet chemotaxis in the transwell chemotaxis and microplate chemotaxis assays, respectively. Interestingly, OVA-sensitization of mice caused significant platelet chemotaxis towards eotaxin compared to sham-sensitized mice, indicating that allergen-sensitization of mice leads to increased CCR3 receptor activity. Other studies investigating the role of CCR3 in allergic inflammation have also shown enhanced activity towards CCR3 ligands of leukocytes from animals sensitized to allergen compared to control groups (Sehmi et al., 2003; Francis et al., 2007; Wang et al., 2007). In eotaxin-1 and eotaxin-2 deficient mice, airway eosinophilia was reduced following allergen challenge in OVA-sensitized mice (Pope et al., 2005). Similarly, eotaxin-1, eotaxin-2 and RANTES expression are increased in the lungs of allergic asthmatic patients (Ying et al., 1999). Eotaxin-1 levels were also elevated in sputum samples from patients with asthma and in the plasma of asthmatic patients experiencing an acute inflammatory response,

compared with stable asthmatic patients (Ying et al., 1997, 1999; Lilly et al., 1999; Yamada et al., 2000). In CCR3 deficient mice, eosinophil recruitment to the lungs following allergen challenge was impaired, and mast cell homing after allergen sensitization and challenge was effected (Humbles et al., 2002). Thus the enhanced activity of the CCR3 receptor on other cell types may also exist on platelets.

The importance of the CCR3 receptor on platelet recruitment and migration *in-vivo*, was investigated. Antagonism of the CCR3 receptor using SB328437, caused a reduction in platelet rolling, platelet adhesion, and platelet accumulation into the cremaster muscle; and platelet accumulation into the lungs, in mice sensitized and exposed to HDM. Exogenous eotaxin administration also caused increased platelet rolling and platelet adhesion events in the cremaster muscle. However the sensitization of mice to HDM had no further effect. This would suggest that the sensitization of mice to allergen does not cause increased CCR3 receptor activity. However it is also possible the dose of eotaxin may have been supramaximal, because the dose of eotaxin was previously chosen to induce extensive leukocyte recruitment and transmigration in an inflammatory murine model, and therefore masking differences between sham-sensitized and HDM-sensitized mice in this assay (Riffo-Vasquez et al., 2012). Further experiments could therefore be conducted to examine a full dose relationship between eotaxin and platelet recruitment, and the influence of allergen sensitization on this phenomenon. These studies do reveal that there is a dependency of platelet CCR3 stimulation on platelet recruitment in the context of allergic inflammation, and that pulmonary platelet recruitment and accumulation in a distinct murine model of allergic inflammation requires platelet stimulation via FcεRI (Pitchford et al., 2008). Whilst the sensitizing allergens are different (OVA vs HDM), and whilst both murine and human platelets have been shown to undergo chemotaxis directly to allergen *in-vitro*, to which the platelet donor is sensitized to, it is plausible in the earlier model that the FcεRI-dependent platelet migration was also dependent on platelet stimulation by chemokines. Therefore the relationship between allergen-sensitization, FcεRI stimulation, and platelet CCR3 activity requires further attention.

Curiously, CCR3 receptor inhibition had no effect on leukocyte migration in the cremaster muscle or PMN cell migration to the lung, following allergen challenge. CCR3 receptors are functionally expressed in a number of cell types including eosinophils, mast cells, Th2 cells and basophils, therefore it was hypothesised CCR3 receptor inhibition would impair the recruitment of leukocytes to the lungs and cremaster muscle following allergen challenge (Sallusto et al., 1997; Ugucioni et al., 1997; Pease et al., 1998; Ochi et al., 1999). A single dose of the CCR3 antagonist SB328437 (30mg/kg) at the time of allergen challenge, may not have been sufficient to effect leukocyte recruitment in our models of allergic inflammation, therefore further experiments elucidating the dose and timing of antagonist treatment should be conducted. Nevertheless, exogenous eotaxin administration caused increased platelet and leukocyte responses to the cremaster muscle, supporting previous literature that CCR3 receptor activation indeed leads to leukocyte recruitment. To conclude, platelet CCR3 receptor expression, *in-vitro* activity and *in-vivo* activity are affected by the sensitization of mice, suggesting an important role for the CCR3 receptor in platelet migration to sites of allergic inflammation.

7.4 Summary

Increased platelet recruitment to the lungs of fatal asthmatic patients, with no evidence of platelet aggregation, suggests a protective inflammatory effect of platelets in asthma. The mechanisms which govern the migration of platelets to sites of allergic inflammation was investigated using *in-vitro* and *in-vivo* assays developed and optimized in this thesis. Platelets were shown to undergo chemotaxis *in-vitro*, and to be recruited and migrate *in-vivo*. Moreover the sensitization of mice to experimental allergen further increased such effects. Platelet chemokine receptors were investigated as a possible mechanism involved in these responses. Platelet CCR3 receptor activity was increased following allergen sensitization, suggesting a possible mechanism through which platelets migrate to sites of allergic inflammation. Interestingly, CXCR4 antagonism was shown to have a biological effect in reducing leukocyte recruitment, yet had no effect on platelet recruitment or migration. Further elucidation of the roles of platelets and chemokine receptors is clearly required, however, the data represented here suggests pulmonary platelet recruitment can occur independently of leukocyte recruitment via different mechanisms involving chemokine stimulation.

7.5 Future work

- 1) Due to a lack of sufficient airway wall tissue, extravascular platelets in the airway walls of healthy, non-fatal asthmatic and fatal asthmatic patients lung biopsy samples could not be quantified, therefore further staining on more sections should be considered.
- 2) As previously mentioned, studies on asthmatic patient lung biopsy samples following antigen exposure in clinically controlled conditions, would aid the difficulties in measuring platelet recruitment to the lungs.
- 3) In the microfluidic chemotaxis assay, further validation experiments to determine the optimum conditions for platelet chemotaxis are required, before the effects of allergen sensitization on platelet chemotaxis can be investigated.
- 4) Investigate conditions under which platelets might transmigrate across a barrier using flow chambers coated with endothelial cell monolayers.
- 5) Use near-infrared whole body imaging to conduct a temporal analysis of platelet vs eosinophil, or leukocyte recruitment to the lungs after allergen exposure, using probes to identify activated platelet and leukocyte populations.
- 6) Investigate whether sensitization to allergen affects megakaryocyte phenotype in the first instance, whether this is subset specific or not, or whether platelet activity is affected on existing platelets.
- 7) In the allergic intravital microscopy model of the cremaster muscle, a dose response to chemokine receptor antagonists and experimenting with the timing of dose, may clarify the effects of platelet chemokine receptors.
- 8) To further investigate the role of the CCR3 receptor *in-vivo*, by performing a dose response to eotaxin, and dissecting and viewing the cremaster muscle at different time intervals post allergen challenge.
- 9) Use chemokine receptor deficient mice to investigate platelet chemokine receptors roles on platelet migration in *in-vitro* and *in-vivo* assays.

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- 10) Study the intracellular pathways connecting platelet chemokine receptor activity and FcεRI receptor stimulation in allergic inflammatory states.

Chapter 8 References

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